Cu\(^{2+}\)-specific CopB transporter: Revising \(P_{1B}\)-type ATPase classification

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The copper-transporting \(P_{1B}\)-ATPases, which play a key role in cellular copper homeostasis, have been divided traditionally into two subfamilies, the \(P_{1B,1}\)-ATPases or CopAas and the \(P_{1B,2}\)-ATPases or CopBs. CopAs selectively export Cu\(^{2+}\) whereas previous studies and bioinformatic analyses have suggested that CopBs are specific for Cu\(^{2+}\) export. Biochemical and spectroscopic characterization of \(S\)phaerobacter thermophilus CopB (S\(St\)CopB) show that, while it does bind Cu\(^{2+}\), the binding site is not the prototypical \(P_{1B,1}\)ATPase transmembrane site and does not involve sulfur coordination as proposed previously. Most important, \(St\)CopB exhibits metal-stimulated ATPase activity in response to Cu\(^{2+}\), but not Cu\(^{2+}\), indicating that it is actually a Cu\(^{2+}\) transporter. X-ray absorption spectroscopic studies indicate that Cu\(^{2+}\) is coordinated by four sulfur ligands, likely derived from conserved cysteine and methionine residues. The histidine-rich N-terminal region of \(St\)CopB is required for maximal activity, but is inhibitory in the presence of divalent metal ions. Finally, reconsideration of the \(P_{1B,1}\)-ATPase classification scheme suggests that the \(P_{1B,1}\) and \(P_{1B,2}\)ATPase subfamilies both comprise Cu\(^{2+}\) transporters. These results are completely consistent with the known presence of only Cu\(^{2+}\) within the reducing environment of the cytoplasm, which should eliminate the need for a Cu\(^{2+}\) \(P_{1B}\)-ATPase.

\(P_{1B}\)-ATPase | copper homeostasis | copper efflux | CopB | CopA

Metal ions are required for critical cellular functions (1). In particular, copper is an essential cofactor in a multitude of proteins, but can be toxic, destroying iron–sulfur clusters and causing oxidative stress (2). Thus, regulating copper concentrations presents a major challenge to all organisms; 44% of the copper proteome has been assigned to copper homeostasis (3, 4). Proteins involved in copper homeostasis include metalloproteins, metallochaperones, and membrane transporters (5). Among the transporters are the \(P_{1B}\)-ATPases, a subset of the \(P\)-type ATPase family (6). \(P_{1B}\)-ATPases are ubiquitous in nature and couple the energy of ATP hydrolysis to translocation of transition metal ions, including Cu\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\)/Cd\(^{2+}\)/Pb\(^{2+}\), and Co\(^{2+}\) (7–9). \(P_{1B}\)ATPases consist of six to eight transmembrane (TM) helices, an ATP-binding domain (ATPBD), an actuator domain, and in many cases, additional soluble metal-binding domains (MBDs), typically at the N terminus. Transport is accomplished via a classical Post-Albers cycle in which phosphorylation of a conserved aspartate residue in the ATPBD causes the enzyme to cycle between high (E1) and low (E2) affinity metal-binding states (10). In humans, defects in the Cu\(^{2+}\)-transporting \(P_{1B}\)-ATPases ATP7A and ATP7B lead to Menkes syndrome and Wilson disease, respectively (11).

\(P_{1B}\)-ATPases have been categorized into seven subtypes (\(P_{1B,1}\), 1B-2, 1B-3, 1B-4, 1B-5, 1B-6, and 1B-7) based on conserved motifs in the TM domain, the presence of different types of MBDs, and biochemical and genetic data linking individual transporters to specific metal ions (7, 12, 13). One of the key TM motifs is a three-residue, cysteine-containing sequence in TM helix 4; other conserved residues in TM helix 6 have also been considered in developing the classification scheme. Of the subclasses, the \(P_{1B,2}\)-ATPases transport Zn\(^{2+}\), Cd\(^{2+}\), and Pb\(^{2+}\) (CPC motif) (9, 14, 15), and the \(P_{1B,4}\)-ATPases transport Co\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\), and Fe\(^{2+}\) (SCA motif) (10–19). The metal specificities of the \(P_{1B,5}\) (PCP motif), \(P_{1B,6}\) (SCA motif), and \(P_{1B,7}\)-ATPases (CSC motif) remain unclear, although some evidence links the \(P_{1B,5}\)-ATPases to Ni\(^{2+}\) and Fe\(^{2+}\) (20, 21). The remaining two groups are the copper transporters. The \(P_{1B,1}\)-ATPases, which include ATP7A and ATP7B, transport Cu\(^{2+}\) (9, 22), whereas the \(P_{1B,3}\)-ATPases are proposed to transport Cu\(^{2+}\) (23, 24). These two subfamilies differ from one another in several ways. First, the TM helix 4 motif is CPC in the \(P_{1B,1}\)-ATPases and CPH in the \(P_{1B,3}\)-ATPases. The presence of this histidine has been widely assumed to confer a preference for Cu\(^{2+}\) (12, 13, 23, 24). The \(P_{1B,1}\)-ATPases, referred to as CopBAs, are unique in that they contain a histidine-rich N-terminal extension that is proposed to be an MBD (23). This extension varies considerably in length, ranging from about 40–120 residues (SI Appendix, Fig. S1). By contrast, the \(P_{1B,1}\)-ATPases, of which many are designated CopAs, usually have MBDs comprising one to six ferredoxin-like domains characterized by a conserved CXXC metal-binding motif that binds a single Cu\(^{2+}\) ion (25).

The \(P_{1B,1}\)-ATPases have been studied extensively due to the link to Wilson and Menkes diseases (11), with multiple solution structures of the MBDs (25), electron microscopy structures of \(A\)rchaoglobus \(f\)ulgidus CopA (26) and ATP7B (27), and a crystal structure of CopA from \(L\)egionella \(p\)neumophila (\(L\)pCopA), albeit in the absence of bound copper or the MBD (28), available. In addition, the roles of the MBDs (29) and the nature of the TM Cu\(^{2+}\)-binding site (9, 29) have been probed by a range of biochemical, biophysical, and functional studies. By contrast, just

Significance

Copper is an important biological cofactor, but can also be toxic in excess. Members of the \(P_{1B}\)-ATPase family of membrane transporters couple the energy of ATP hydrolysis to translocation of metal ions across membranes. \(P_{1B}\)-ATPases have been classified into groups on the basis of sequence and metal ion specificity. Two subfamilies, the \(P_{1B,7}\)-ATPases, which are linked to human diseases of copper metabolism, and the \(P_{1B,3}\)-ATPases, found only in bacteria, have been assigned as Cu\(^{2+}\) and Cu\(^{2+}\) transporters, respectively. Here we show that the \(P_{1B,5}\)-ATPases are actually Cu\(^{2+}\) transporters, necessitating revision of the classification scheme. These findings are consistent with the presence of only Cu\(^{2+}\) in the cytoplasm, which eliminates the need for a Cu\(^{2+}\) efflux pump.


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a few studies of the P1B,3-ATPases, which are found only in bacteria, have been reported. While early work on Enterococcus hirae CopB (EhCopB) (30) suggested that it is a Cu⁺ transporter, subsequent studies of thermophilic CopBs from A. fulgidus (AfCopB) (23) and Thermus thermophilus (TtCopB) (31) indicated that CopBs are mainly Cu⁺ transporters. Metal binding by the histidine-rich N-terminal region has not been investigated for any CopB. Thus, it remains unclear how the two classes of copper P1B,3-ATPases confer selectivity for Cu⁺ versus Cu²⁺.

To investigate the basis for discrimination between Cu⁺ and Cu²⁺ by P1B,3-ATPases, we biochemically and spectroscopically characterized the CopB from Sphaero bacter thermophilus (StCopB), both with and without its 120-residue N-terminal histidine-rich domain. Contrary to prior reports and accepted dogma, our results indicate that StCopB is a Cu⁺ transporter. Binding of Cu²⁺ is observed, but mutagenesis and electron paramagnetic resonance (EPR) spectroscopic data indicate that it is not located in the proposed TM binding site. Instead, X-ray absorption spectroscopic (XAS) studies on the Cu⁺-bound protein define a sulfur-based coordination environment in the TM region. A reexamination of the bioinformatics analysis suggests that the P1B,1- and P1B,3-ATPases are subsets of the same class and provides a revised framework for overall P1B,3-ATPase classification. Finally, insights into the possible role of the N-terminal histidine-rich region are presented.

Results and Discussion

StCopB Does Not Bind Cu²⁺ in the Archetypal P1B,3-type ATPase TM Site. We initially assumed that StCopB is a Cu²⁺ transporter, as has been reported for AfCopB (23) and TtCopB (31). To probe Cu²⁺ binding by the CopB TM metal-binding site and to investigate whether the N-terminal histidine-rich regions bind metal ions, we expressed and purified both full-length StCopB (WT-StCopB, residues 1–785) and StCopB lacking the N-terminal region (ΔMBD-StCopB, residues 114–785) (SI Appendix, Fig. S2). After overnight incubation with varying amounts of Cu²⁺, followed by removal of excess copper, quantitation indicated that ΔMBD-StCopB binds a single Cu²⁺ ion even when incubated with a 10-fold excess (SI Appendix, Table S1). We first believed the Cu²⁺ to be bound at the TM-binding site reported in a recent study of AfCopB (24). Indeed, the EPR spectrum of ΔMBD-StCopB exhibited the type-2 Cu²⁺ signature (Fig. 1) reported for AfCopB (g⊥ = 2.23, g∥ = 2.06, 4A1 = 560 MHz for ΔMBD-StCopB; g⊥ = 2.23, g∥ = 2.06, 4A1 = 565 MHz for AfCopB) and attributed to nitrogen/oxygen (N/O) equatorial ligands on the basis of the Peisach–Blumberg correlation diagram (32). Consistent with this environment, electron-nuclear double resonance spectra collected on ΔMBD-StCopB identified at least one directly coordinated equatorial =N ligand and suggested the presence of a coordinated H₂O (SI Appendix, Fig. S3A).

Potential ligands to this presumed TM Cu²⁺-binding site were identified by aligning a homology model of WT-StCopB (23) with the crystal structure of LpCopA (28). In this model, the CPH motif of StCopB is in the same position as the CPC motif of LpCopA (SI Appendix, Fig. S4). To assess whether the Cu²⁺-binding site involves the CPH motif, as suggested for AfCopB (24), several variants (C404A, H406A, and C404A, H406A) of ΔMBD-StCopB were generated. Surprisingly, the EPR spectra of all three variants exhibited the same signal as ΔMBD-StCopB (SI Appendix, Fig. S3B), showing that the CPH motif is not the site of Cu²⁺ binding in StCopB.

We then investigated the Cu²⁺-binding properties of full-length WT-StCopB. Reconstitution of WT-StCopB with increasing amounts of Cu²⁺ (0–15 equivalent [equiv]) indicated that it can bind up to approximately eight Cu²⁺ ions (SI Appendix, Fig. S5). Since ΔMBD-StCopB can bind approximately one Cu²⁺ ion, no fewer than seven Cu²⁺ ions must bind to the MBD. In fact, none of the spectra at loading concentrations between 0.75 and 10 Cu²⁺ equiv show the spectrum of ΔMBD-StCopB (SI Appendix, Fig. S3C). Instead, WT-StCopB loaded with 0.75 equiv Cu²⁺ exhibits a type-2 Cu²⁺ EPR signal (g⊥ = 2.25, g∥ = 2.05, 4A1 = 565 MHz) similar to, but distinct from, that of ΔMBD-StCopB, in particular with resolved ¹⁴N hyperfine along g∥ (Fig. 1). The WT-StCopB spectrum shows a strong resemblance to that of Cu²⁺-imidazole (34). This similarity, combined with the abundance of histidine residues in the MBD, as well as the fact that this spectrum is distinct from that of ΔMBD-StCopB, suggests that histidine side chains in the MBD HXXH motifs (SI Appendix, Fig. S1) are the probable WT-StCopB Cu²⁺ ligands. Increasing the amount of Cu²⁺ increases the intensity of this signal without changing the Cu²⁺ g⊥ and 4A1 values, while the ¹⁴N hyperfine splitting along g∥ is lost upon addition of 5 equiv Cu²⁺. This observation indicates that the multiple Cu²⁺ sites in the MBD have very similar coordination spheres with similar sets of N/O ligands, but with slight variations in their imposed coordination geometries.

Diversity Among CopB MBDs and Characterization of the StCopB MBD. Sequence alignments show that CopB-MBD sequences are diverse in length and sequence (SI Appendix, Fig. S1). A close examination of these sequences reveals a strong resemblance to hydrophilins, proteins characterized by high glycine content (>6%) and a high hydrophilicity index (>1.0) (35). The StCopB-MBD sequence, which is composed of ~27% histidine residues located in repeated HXXH and HXH motifs, has >13% glycine residues and is highly hydrophilic with a grand average of hydropathicity (36) score of −1.5. Polar ϕ-segments are present throughout the StCopB MBD, although the canonical hydropolin (Y-, S-, and K-) segments (37) are largely absent, similar to the histidine-rich plant dehydrins, which are involved in protecting cells from a variety of stress conditions, particularly osmotic and toxic metal stress (38).

To probe metal binding by the isolated StCopB MBD, recombinantly expressed StCopB MBD (residues 1–120, without added histidine tag) was purified using a Ni-NTA column (SI Appendix, Fig. S2). Consistent with secondary and tertiary structure predictions (SI Appendix, Fig. S6), the circular dichroism (CD) spectrum...
of StCopB MBD exhibits a large negative signal at 198 nm, indicative of random coil secondary structure (SI Appendix, Fig. S7). Addition of one equivalent of metal (Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), or Ag\(^{+}\)) did not produce any spectral changes. Further metal addition led to protein precipitation, reminiscent of the metal-induced aggregation reported for histidine-rich plant dehydrins (39). The CD spectrum collected in the presence of 33\% 2,2,2-trifluoroethanol, known to stabilize secondary structure in proteins (40, 41), showed high helical content (208 and 222 nm), suggesting that StCopB MBD can form an ordered secondary structure (SI Appendix, Fig. S7). In addition, homology modeling of WT-StCopB predicts multiple different folds for the MBD within WT-StCopB, including a small ferredoxin-like fold or several helices (SI Appendix, Fig. S6). Thus, it might adopt a folded structure in the context of intact StCopB.

StCopB Is a Cu\(^{2+}\)-Specific P\(_{i}\)-ATPase. We next measured the ATP hydrolysis activity of WT-StCopB and ΔMBD-StCopB in the presence of various divalent metal ions (Cu\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), or Zn\(^{2+}\)). The majority of previously characterized P\(_{i}\)-ATPases, including AaCopB, TtCopB, and a CopB from *Aquifex aeolicus* (AaCtrA3), have been implicated in Cu\(^{2+}\) transport (23, 31, 42). Surprisingly, for both WT-StCopB and ΔMBD-StCopB, Cu\(^{2+}\)-dependent stimulation of ATPase activity was not observed (Fig. 2A). Moreover, the basal ATPase activity for WT-StCopB was inhibited at ≥10 \(\mu\)M Cu\(^{2+}\), whereas Cu\(^{2+}\) addition did not affect the ΔMBD-StCopB basal activity. A similar effect was observed upon addition of Zn\(^{2+}\) to WT-StCopB and ΔMBD-StCopB (Fig. 2A). The observed inhibition could be due to binding of excess Cu\(^{2+}\) and Zn\(^{2+}\) by WT-StCopB (SI Appendix, Fig. S5). Conformational changes in the MBD might then hinder ATP hydrolysis by the ATPBD. WT-StCopB also exhibited some activity in the presence of Co\(^{2+}\), but not in the presence of Ag\(^{+}\), whereas ΔMBD-StCopB displayed the opposite trend with no activity stimulation by Co\(^{2+}\) and some activity upon Ag\(^{+}\) addition.

Stimulation of ATPase activity and transport by Ag\(^{+}\) has been reported for AtCopB (23) and EhCopB (30).

Since early studies of EhCopB indicated that it is a Cu\(^{2+}\) transporter (30), and since three other CopBs [AtCopB (23), TtCopB (31), and AaCtrA3 (42)] were in fact significantly active in the presence of Cu\(^{2+}\) as well as Cu\(^{2+}\) (25, 50, and 50\% of Cu\(^{2+}\)-stimulated activity, respectively), we considered the possibility that StCopB may be specific for Cu\(^{2+}\) rather than Cu\(^{2+}\) and performed the ATPase activity assay in the presence of Cu\(^{2+}\) generated by reduction of Cu\(^{2+}\) with 2-mercaptoethanol (2-ME). Unexpectedly, both WT-StCopB and ΔMBD-StCopB showed significant Cu\(^{2+}\)-stimulated ATPase activity with maximal values of 123 ± 17 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\) (basal activity: 42.5 ± 7.2 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\)) and 50 ± 6 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\) (basal activity: 18.8 ± 2.0 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\)) respectively, at 55 °C, which is the optimal growth temperature for the bacteria (43) (Fig. 2A, Inset). Lipids (0.01% asolectin) were required to observe this metal-stimulated ATPase activity. The measured \(K_m\) values of 1.6 ± 0.3 \(\mu\)M and 2.5 ± 0.6 \(\mu\)M for Cu\(^{2+}\) for WT-StCopB and ΔMBD-StCopB (Fig. 2B), respectively, are similar to previously reported \(K_m\) values for CopB and CopA (31, 44, 45). Furthermore, Cu\(^{2+}\)-stimulated activity was eradicated by 1 mM bathocuproinedisulfonic acid (BCS) (Fig. 2A), a high-affinity Cu\(^{2+}\)-specific chelator. The pronounced activity enhancement in the presence of Cu\(^{2+}\) compared with all other metal ions tested indicates that StCopB is a Cu\(^{2+}\)-specific transporter.

Having demonstrated that Cu\(^{2+}\) does not bind in the CPH TM site, we reasoned that this site might instead bind Cu\(^{+}\). Consistent with this hypothesis, the three CPH motif variants (C404A, H406A, and C404A,H406A) did not exhibit Cu\(^{2+}\)-stimulated ATPase activity (Fig. 2C). These results are at least in part due to disrupted binding of Cu\(^{2+}\). Whereas WT-StCopB and ΔMBD-StCopB bind ~1 molar equiv of Cu\(^{2+}\), consistent with a single high-affinity Cu\(^{2+}\)-binding site and no Cu\(^{2+}\) binding by the MBD.

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**Fig. 2.** Functional characterization of StCopB. (A) Metal-stimulated ATPase activity (nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\)) of WT-StCopB and ΔMBD-StCopB in the presence of 10 \(\mu\)M metal ions. Activity levels were corrected against basal activity in the absence of metal ions (WT-StCopB basal activity: 42.5 ± 7.2 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\) and ΔMBD-StCopB basal activity: 18.8 ± 2.0 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\)). The maximal ATPase activity was observed in the presence of 10 \(\mu\)M Cu\(^{2+}\) (WT-StCopB: 123 ± 17 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\); ΔMBD-StCopB: 50 ± 6 nmol P\(_{i}\) mg\(^{-1}\) min\(^{-1}\)). (Inset) Maximal Cu\(^{2+}\)-stimulated WT-StCopB ATPase activity (basal corrected) at 37 °C, 55 °C, and 75 °C (representative values shown for 37 °C and 75 °C). (B) Specific ATPase activity (basal-corrected) of WT-StCopB (circles) and ΔMBD-StCopB (triangles) as a function of Cu\(^{2+}\) concentration (\(\mu\)M) fitted to the equation \(y = (\text{max} \times x \cdot y)/(x + K_{\text{eq}})\). (C) Basal and Cu\(^{2+}\)-stimulated ATPase activity levels of ΔMBD-StCopB and its variants. (D) Relative effects of various reducing agents (DTT, cysteine, ascorbate) on maximal Cu\(^{2+}\)-stimulated ATP hydrolysis activity of WT-StCopB and ΔMBD-StCopB. Additionally, almost complete inhibition of ATPase activity was observed in the presence of 1 mM sodium orthovanadate, a phosphate analog and P-type ATPase competitive inhibitor. In all cases, error bars represent the SD of the average of at least three independent experiments.
(SI Appendix, Table S2), Cu⁺ binding by AMBD-StCopB variants C404A, H406A, and C404A,H406A is diminished by ~20–50%. Mutation of the conserved methionine in the TM helix 6 MSXST (12, 13) motif (M739A) also abolished activity, similar to what was observed for the LpCopA M717V variant (46), although the M739A variant still bound close to 1 equiv of Cu⁺. The lack of ATPase activity likely results from disruption of the ion release pathway as proposed for LpCopA (46). Surprisingly, replacement of the conserved histidine residue in the CPH motif, H406, with cysteine to mimic the CPC motif in the Cu⁺-transporting P₁,B₁-ATPases resulted in similar basal ATPase activity to ΔMBD-StCopB, but absolutely no activity in the presence of Cu⁺. However, this H406C variant can be reconstituted with ~2.1 molar equiv of Cu⁺ (SI Appendix, Table S2), suggesting that conversion of the CPH motif to a CPC motif introduces a second Cu⁺-binding site.

To probe the apparent difference in metal selectivity between A/CopB and StCopB, we purified full-length A/CopB (residues 1–690) (SI Appendix, Fig. S2) and tested it for Cu⁺-stimulated activity. Similar to what was reported by Meloni et al. (24), we observed no Cu⁺- or Cu⁺²-stimulated activity for purified A/CopB even in the presence of the lipids (0.01%) asolectin. A Vₘₐₓ value of 1.2 μmol P_i mg⁻¹ h⁻¹ (20 μmol P_i mg⁻¹ min⁻¹) was reported for A/CopB in the presence of 1 μM CuCl₂, but may represent basal activity (24). We also tested A/CopB in Escherichia coli membranes prepared without any purification and detergent solubilization [similar to the procedure reported by Mana-Capelli et al. (23)] and observed very high basal activity at 75 °C with no detectable metal stimulation.

To further compare our results to previous work, we investigated the use of reductants other than 2-ME to generate Cu⁺, specifically DTT and cysteine, which were used in other studies of CopAs (28, 44) and CopBs (24). In contrast to 2-ME, no Cu⁺- or Cu⁺²-stimulated ATPase activity for WT-StCopB or ΔMBD-StCopB was observed in the presence of DTT or cysteine (Fig. 2D). Similar levels of Cu⁺-stimulated ATPase activity were observed in the presence of ascorbate for ΔMBD-StCopB, but no stimulation was observed for WT-StCopB, which could be due to Cu⁺⁻ascorbate-mediated oxidation of the histidine residues in the MBD (47). It is important to note that DTT and cysteine can coordinate Cu⁺⁻DTT and cysteine, maintaining a Cu⁴⁺ coordination within the TM region, we performed XAS analysis of Cu⁰-MBD (47). It is important to note that DTT and cysteine can coordinate Cu⁰ within the TM region, as proposed for A/CopBs (28, 44) and CopBs (24). In contrast to 2-ME, no Cu⁺-stimulated ATPase activity was detected. The EXAFS data suggest a TM Cu⁺⁻binding site. Similar to what was reported by Meloni et al. (23), we confirmed the complexity of Cu⁺⁻DTT and cysteine by monitoring Cu⁺ chelation with BCS at ~485 nm (SI Appendix, Fig. S8). Immediate Cu⁺⁻BCS complex formation was observed upon addition of 2-ME or ascorbate, whereas reaction with DTT, cysteine, and tris(2-carboxyethyl)phosphine (TCEP) for 15 min failed to produce any significant Cu⁺⁻BCS complex, indicating that Cu⁺ is either sequestered by these reductants (DTT, cysteine) or not reduced (TCEP) and thus would not be available to stimulate ATPase activity. These findings explain the relative lack of Cu⁺-stimulated activity observed for A/CopB (23) and TtCopB (31), which were assayed in the presence of DTT. TtCopB was in fact suggested to use Cu⁺ in vivo (31).

A Sulfur-Containing Cu⁺ Coordination Environment. To elucidate the atomic details of StCopB Cu⁺ coordination within the TM region, we performed XAS analysis of Cu⁺⁻loaded WT-StCopB. The WT-StCopB XANES spectrum exhibits a 1s→4p transition at 8,983 eV (Fig. 3A), consistent with the presence of Cu⁰. Cu K-edge extended X-ray absorption fine structure (EXAFS) data of the Cu⁺⁻loaded WT-StCopB (Fig. 3B) were best fit with a nearest-neighbor environment composed of three S ligands at an average bond length of 2.27 Å and one S ligand at 2.48 Å, with no long-range scattering observed (SI Appendix, Table S3). There is no evidence for histidine coordination. Close inspection of the homology model and sequence alignment indicates the presence of a sulfur-lined inner channel that could be involved in Cu⁺⁻transport (Fig. 4A). The EXAFS data suggest a TM Cu⁺⁻binding site coordinated by Cys⁴₀⁴ and potentially three methionine residues (Met¹₈₆, Met¹₇₇, Met²₂₂) in a tetrahedral fashion, although the presence of a chloride ligand cannot be ruled out. Similar sites involving Cu⁺⁻coordinated by Cys and Met residues in a tridimensional geometry have been proposed for LpCopA (50) and human CTR1 (51). Importantly, all four proposed ligands are absolutely conserved among CopBs, with the methionine residues deriving from a PGMM motif in TM helix 1 and a MLLG motif in TM helix 2 (Fig. 4). These motifs, rather than the CopB CPH motif, likely confer metal specificity for Cu⁺⁻.

Comparison of CopB and CopA sequences indicates that many key residues proposed for Cu⁺⁻transport by LpCopA (50, 52) are highly conserved among CopBs as well (Fig. 4B). In particular, a channel lined with residues Met¹₄₈, Met¹₇₇, Glu¹₈₉, Glu²₀₅, and Asp³₃₇ in LpCopA corresponds to a proposed channel lined with Met¹₈₆, Met¹₇₇, Glu²₁₆, Glu₂₃₁, and Asp³₆₂ in StCopB and is absolutely conserved among CopB and CopA sequences. In addition, StCopB Met²₂₂ is within the putative metal transport path and is strictly conserved among CopBs, but not in CopAs. This residue could potentially serve as a ligand in the absence of the second cysteine present in the CopA CPC motif, but not in the CopB CPH motif. The inhibitory effect of Cu⁺⁻on the CPC StCopB mutant might therefore derive from Cu⁺⁻binding in an incorrect conformation involving the second cysteine. These comparisons suggest that CopA and CopB utilize similar, but not identical, sets of residues to bind and export Cu⁺⁻.

Finally, we probed the copper coordination environment of Cu⁺⁻⁻loaded ΔMBD-StCopB and the TM helix 4 CPH motif variants by XAS (SI Appendix, Fig. S9 and Table S4). Both the C404A and H406A variants displayed coordination environments similar to ΔMBD-StCopB, involving only N/O ligands and no S ligands. EXAFS fits with 3 N/O and 1 S ligands led to high Debye–Waller factors, indicating that an S ligand is not involved in Cu⁺⁻⁴⁻binding. This result is in contrast to what was observed for A/CopB (24), but is consistent with the EPR analysis of these proteins (Fig. 1A) as well as that of A/CopB (24). Additionally, the signature camellia fragment observed in the Cu⁺⁻⁻loaded ΔMBD-StCopB EXAFS indicates histidine ligation.

Revising the P₁,B⁻ATPase Classification Scheme. Given the strong evidence that StCopB and likely the other characterized CopBs are actually Cu⁺⁻transporters, we reexamined the classification of P₁,β⁻⁻ATPases into the P₁,β⁻⁻A¹⁻β⁻⁻subfamilies (7, 12, 13). Notably, we identified several hundred P₁,β⁻⁻ATPase sequences that belong to a subgroup characterized by a conserved CPG motif in TM helix 4. No member of this P₁,β⁻⁻ATPase subfamily has been characterized. We then reinvestigated our previously generated
Conclusions. The combined results indicate that $S\text{t} \text{CopB}$ is a Cu$^{2+}$ transporter that binds a single Cu$^{2+}$ ion in the TM region using four sulfur ligands. It does bind Cu$^{2+}$, but the mutagenesis data show that the TM helix 4 CPH motif as hypothesized previously. Instead, EPR and EXAFS data indicate that Cu$^{2+}$ is coordinated by N/O ligands, including histidine. The location of the Cu$^{2+}$-binding site remains unclear. The activity data clearly show that Cu$^{2+}$, but not Cu$^{+}$, stimulated ATP hydrolysis by $S\text{t} \text{CopB}$, and previous reports of no Cu$^{2+}$-stimulated activity can be attributed to the use of Cu$^{2+}$-complexing agents as reductants. While the hydrophilin-like $S\text{t} \text{CopB}$ MBD is necessary for maximal activity, it surprisingly does not bind Cu$^{2+}$. Instead, binding of approximately eight Cu$^{2+}$ or Zn$^{2+}$ ions inhibits basal ATPase activity, perhaps by interfering with the ATPBD. Thus, the MBD may have multiple functions, stabilizing the ATPBD during Cu$^{2+}$ efflux and sequestering metal ions other than Cu$^{2+}$ under conditions of stress, as observed in the similar plant dehydrins (38). The overall conservation of residues proposed to be important for copper transport by CopAs and CopBs is striking, and, taken together with the revised sequence similarity network, suggests that these two $P_{1B}$-ATPase subfamilies represent related solutions for Cu transport rather than the existence of specific Cu$^{2+}$ and Cu$^{+}$ transporters. This conclusion resolves a long-standing conundrum in the field: copper within the reducing environment of the cytoplasm should be Cu$^{2+}$ (4, 31, 53, 54), obviating the need for a Cu$^{2+}$-$P_{1B}$-ATPase.

Materials and Methods

Detailed procedures for preparation of $S\text{t} \text{CopB}$ proteins and variants, protein metal loading and quantitation, and ATPase activity assays are included in SI Appendix, SI Materials and Methods. Also described are computational methods for structure prediction, homology modeling, and sequence containing sequences clustered strongly within the $P_{1B}$-2 subfamily, suggesting that these sequences are likely part of that subfamily rather than a separate subfamily.

Fig. 4. Proposed $S\text{t} \text{CopB}$ Cu$^{2+}$-binding site. (A) Close-up view of potential Cu$^{2+}$-binding site in the $S\text{t} \text{CopB}$ homology model. Residues that may be important in Cu$^{2+}$ binding and are absolutely conserved among CopBs include Met186 (TM1), Met187 (TM1), Met224 (TM2), and Cys404 (TM4). (B) Sequence alignment of the TM domains of human ATP7A, L. pneumophila CopA (LpCopA), E. hirae CopB (EhCopB), A. aeolicus copper transporter (AaCra3), A. fulgidus CopB (AfCopB), Methanosarcina acetivorans CopB (MaCopB), S. thermophilus CopB (StCopB), T. thermophilus CopB (TtCopB), and Nostoc sp. CopB (NsCopB) showing absolutely conserved residues (blue) among CopAs and CopBs. The residues absolutely conserved among CopBs are also highlighted (*).

$P_{1B}$-ATPase sequence similarity network (13), incorporating 250 of these CPG-containing sequences and 22 sequences containing the SCSC TM helix 4 motif (previously classified as $P_{1B}$-7-ATPases) into the clustering analysis (Fig. 5). Similar to the previous analysis, the sequences containing different TM helix 4 motifs clustered into distinct groups. The $P_{1B}$-5 (PCP) and $P_{1B}$-6 (SCA) subfamilies clustered separately, as previously observed. The CPC motif-containing sequences clustered strongly together, with some connections to the $P_{1B}$-2 sequences containing the CPC motif. Importantly, the $P_{1B}$-1 (CPC) and $P_{1B}$-3 (CPH) subfamilies clustered separately as shown previously (13), but they are strongly connected when the network is visualized at 35% sequence identity, indicating that the two subfamilies are closely related. Moreover, they are more closely related to each other than both $P_{1B}$-1 and $P_{1B}$-2 sequences that contain the CPC motif. Finally, the SCSC motif-

Fig. 5. The extended $P_{1B}$-ATPase similarity network. Sequences are represented as nodes (colored circles), and the strength of their similarity is indicated by edges (lines connecting colored circles). Sequences are color coded and labeled by their signature TM helix 4 motifs. The pink cluster represents newly identified sequences containing a conserved CPC motif. Representative CopAs and CopBs discussed in the text are labeled.
neutrality similarity network generation. Standard methods were used for collection of circular dichroism, EPR, and XAS spectroscopic data; instrument specifics are given in SI Appendix, SI Materials and Methods.

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