Archaeoglobus fulgidus CopB Is a Thermophilic Cu\(^{2+}\)-ATPase

FUNCTIONAL ROLE OF ITS HISTIDINE-RICH N-TERMINAL METAL BINDING DOMAIN*

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\(\text{P}_{1}\text{b}\)-type ATPases transport heavy metal ions across cellular membranes. \(\text{Archaeoglobus fulgidus}\) CopB is a member of this subfamily. We have cloned, expressed, in \(\text{Escherichia coli}\), and functionally characterized this enzyme. CopB and its homologs are distinguished by a metal binding sequence Cys-Pro-His in their sixth transmembrane segment (H6) and a His-rich N-terminal metal binding domain (His-N-MBD). CopB is a thermophilic protein active at 75 °C and high ionic strength. It is activated by Cu\(^{2+}\) with high apparent affinity \((K_{d} = 0.28 \mu M)\) and partially by Cu\(^{+}\) and Ag\(^{+}\) (22 and 55%, respectively). The higher turnover was associated with a faster phosphorylation rate in the presence of Cu\(^{2+}\). A truncated CopB lacking the first 54 amino acids was constructed to characterize the His-N-MBD. This enzyme showed reduced ATPase activity (50% of wild type) but no changes in metal selectivity, ATP dependence, or phosphorylation levels. However, a slower rate of dephosphorylation of the \(E_{P}(\text{Cu}^{2+})\) form was observed for truncated CopB. The data suggest that the presence of the His residue in the putative transmembrane metal binding site of CopB determines a selectivity for this enzyme that is different for that observed in Cu\(^+/Ag^{+}\)-ATPases carrying a Cys-Pro-Cys sequence. The His-N-MBD appears to have a regulatory role affecting the metal transport rate by controlling the metal release/ dephosphorylation rates.

Heavy metal transport ATPases, also called CPX-ATPases, belong to the \(\text{P}_{1}\text{b}\)-subgroup of the P-type ATPases (1, 2). These enzymes transport transition metal ions such as Cu\(^{2+}\), Ag\(^{+}\), Zn\(^{2+}\), Pb\(^{2+}\), Cd\(^{2+}\), and Co\(^{2+}\). They are found in archaea, bacteria, and eukaryotes where they confer metal tolerance and contribute to metal homeostasis (4–6). Different physiological and biochemical aspects of these enzyme functions have been characterized in some detail. For example, metal-dependent ATPase activity, metal transport capability, inhibition by vanadate, interaction of ATP with high and low affinity, phosphorylation of the invariant Asp in the DKTGT sequence, and distinct dephosphorylation kinetics for \(E_{P}\) and \(E_{D}\) forms have been determined in different \(\text{P}_{1}\text{b}\)-type ATPases (3, 7, 8). Consequently, it is assumed that these enzymes have a catalytic mechanism similar to that observed in well characterized \(\text{P}_{1}\)-type ATPases (Ca-ATPase, Na,K-ATPase, H-ATPase, and so forth) (15, 16). Structurally, most \(\text{P}_{1}\text{b}\)-type ATPases have eight transmembrane segments with a large cytoplasmic loop located between their sixth (H6) and seventh (H7) transmembrane segments (17, 18). They all contain a putative transmembrane metal binding site characterized by “Cys-Pro-X sequences” (Cys-Pro-Cys, Cys-Pro-Ser, Cys-Pro-His, Thr-Pro-Cys, or Ser-Pro-Cys) (4, 5). In addition, many of them contain cytoplasmic metal binding domains in their N terminus (N-MBD) (6, 19, 20). However, significant aspects of these heavy metal transport ATPases remain unclear. Among these and perhaps the most intriguing is how the specificity of these enzymes for different heavy metals is determined. P-type ATPases with different metal specificities have been described. For instance, \(\text{Archaeoglobus fulgidus}\) CopA (8) and \(\text{Escherichia coli}\) CopA (7) are activated by Cu\(^{2+}\) and Ag\(^{+}\), selectivity for Zn\(^{2+}\) (and divalent metals Cd\(^{2+}\) and Pb\(^{2+}\)) has been observed in \(\text{E. coli}\) ZntA (10, 11) and \(\text{Staphylococcus aureus}\) (1258) CadA (12, 13) among others, and \(\text{Synechocystis}\) PCC6803 CoaT seems to be involved in Cu\(^{2+}\) transport (21). The typical Cys-Pro-X sequences located in H6 are probably involved in metal coordination during transport. In support of this idea, the replacement of Cys in the Cys-Pro-Cys of \(\text{E. coli}\) CopA resulted in the loss of copper resistance, transport, and phosphoenzyme formation (7). Similar replacements in \(\text{A. fulgidus}\) CopA produced inactive enzymes that bind the nucleotide but are unable to form the phosphorylated intermediate in the presence of Cu\(^{+}\) and ATP (22). However, it is puzzling that enzymes transporting either monovalent (Cu\(^{+}\) and Ag\(^{+}\)) or divalent (Zn\(^{2+}\), Cd\(^{2+}\), and Pb\(^{2+}\)) metals all carry the Cys-Pro-Cys in their H6 transmembrane segment (7, 8, 10, 23, 25). Similarly, it is difficult to understand how enzymes carrying Cys-Pro-His sequences might transport primarily Cu\(^{+}\) or Ag\(^{+}\) as those containing Cys-Pro-Cys (7, 8, 14). To explain these findings, we recently proposed that residues in transmembranes H7 and H8 might also participate in determining the selectivity of these enzymes. After analyzing the conserved residues in H6, H7, and H8, we proposed that \(\text{P}_{1}\text{b}\)-type ATPases could be subdivided into five subgroups (\(\text{P}_{1b1.a}\)-P\(_{1b5.a}\)) with defined conserved residues and therefore distinct metal selectivity. Among them, 16 enzymes in subgroup \(\text{P}_{1b1.a}\) are characterized by singular invariant residues in the transmembrane region: Cys-Pro-His in H6, Tyr-Asn in H7, and MSXST in H8 (Fig. 1). In addition, these proteins have a His-rich N-terminal MBD (His-N-MBD). Considering the presence of His in these putative metal binding sites and the preference of imidazolium groups for Cu\(^{2+}\) (26), we proposed that these proteins are Cu\(^{2+}\)-transporting ATPases (22). Only one enzyme from this subgroup has been characterized, \(\text{Enterococcus hirae}\).
CopB (27). Studies of *E. hirae* using an *E. hirae* strain lacking the Cu\(^{2+}\)-ATPase CopA have proposed that CopB can drive the outward transport of Cu\(^{2+}\) and Ag\(^{+}\) (14). No Cu\(^{2+}\) transport was established in this system.

The functional role of the His-N-MBD has not been explored. Most P\(_{1,3}\)-type ATPases carry one or more N-terminal metal binding domains characterized by a consensus sequence CXXC (Cys-N-MBD). Studies have shown that the Cys-N-MBDs are not essential for enzymatic activity and probably play a regulatory role (24, 28–30). Lutsenko and co-workers (31) have shown that the isolated Cys-N-MBDs from the Wilson protein interact with the isolated large cytoplasmic loop of the enzyme in a Cu\(^{2+}\)-dependent manner. We have observed that removal of the metal binding capacity from *A. fulgidus* CopA Cys-N-MBD leads to a reduction in the rate of metal release/dephosphorylation steps. Based on these findings, we proposed that the Cys-N-MBDs regulate enzyme turnover by controlling these rate-limiting steps via its interaction with the large cytoplasmic loop. It is then tempting to speculate that the His-N-MBDs have a similar role in the P\(_{1,3}\)-type ATPases.

In this work, we present the characterization of the thermophilic *A. fulgidus* CopB. This is a member of the P\(_{1,3}\)-subgroup with a Cys-Pro-His sequence in H6 and 17 His residues within the first 51 amino acids (Fig. 1). Similar to *A. fulgidus* CopA (8), its expression in *E. coli*, suitable purification, and lack of background activity in membrane preparations (because most determinations are done at 75 °C) facilitate functional studies and allow a detailed examination of different partial reactions. Our studies show that (a) enzymes in the P\(_{1,3}\)-subgroup are Cu\(^{2+}\)-ATPases and (b) their His-N-MBDs are regulatory domains that allow faster enzyme turnover by controlling the rate of metal release.

**EXPERIMENTAL PROCEDURES**

Cloning and Expression—The gene AF0152 from the *A. fulgidus* genome (33) was amplified by polymerase chain reaction using genomic DNA (ATCC, Manassas, VA) as template. The resulting 2070-bp cDNA was cloned into the pBADTOPhis vector (Invitrogen). This vector introduces a C-terminal hexahistidine tag and a V5-epitope suitable for immunodetection. In addition, a cDNA encoding for a truncated form of CopB lacking the N-terminal 54 amino acids was generated by polymerase chain reaction using the full-length cDNA as template and as primers 5’-ATGGAGGACTTCAAGAAGCGATTCTA-3’ and 5’-CCG-CAGCAATCTGGCATTAATGGCCAC-3’. Cells were grown at 37 °C in 2X YT medium (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% in all of our determinations. Background phosphoenzyme measured in the presence of 5 mM EDTA and performed at 37 °C. This temperature, enzyme turn-over is minimum and, in the presence of ATP and outwardly trans-}

**RESULTS**

Among P\(_{1,3}\)-type ATPases, a subset of these enzymes is characterized by the Cys-Pro-His sequence in H6 and a long His-N-MBD (Fig. 1). This subgroup, termed P\(_{1,3}\)-like, includes 16 highly homologous sequences (Fig. 1A). Analysis of their trans-
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Fig. 1. CopB sequence analysis. A, phylogenetic tree of the 16 homologous proteins in the P\textsubscript{1}-subgroup of P-type ATPases (GenBank\textsuperscript{TM} or Swiss-Protein database accession number, genus species, gene name): AAN00136, Streptococcus agalactiae, SAG1262; Q9369, E. coli, HRA-1; AAO03723, Staphylococcus epidermidis, SE0126; Q59370, E. coli, HRA-2; BAC13677, Oceanobacillus iheyensis, OB1721; Q8NLJ0, Corynebacet-
**E. coli** Membrane fractions from untransformed **E. coli** (lanes 1 and 4) and membrane fractions of arabinose-induced **E. coli** transformed with pBAD/TOPO-CopB (lanes 2 and 5) or pBAD/TOPO-T-CopB (lanes 3 and 6) are shown. Lanes 1–3, Coomassie Brilliant Blue-stained gel; lanes 4–6, blot immunostained with anti-(His)₆-kerosidase peroxidase antibodies.

Membrane segment H6 shows that the Cys-Pro-His sequence is fully conserved as are the N(X)₂GYN(X)₄P consensus in H7 and the P(X)₄MSXST(X)₄N in H8 (Fig. 1B). Although they resemble those amino acids conserved in Cu²⁺/Ag⁺-transporting enzymes (**A. fulgidus** CopA, **E. coli** CopA, Menkes and Wilson disease proteins, and so forth), the presence of His in H6 and an additional putative coordinating group in H8 suggests an alternative specificity for the P₁₈₃₉ subgroup of enzymes. To test this hypothesis, we chose to study the small (690 amino acids) thermophilic CopB from **A. fulgidus** (NCBI Protein Data Base accession number AAB91079 and Swiss Protein Data Base accession number O30085). This is a typical P₁₈₃₉-group ATPase with a high identity percent (38–70%) with other members of the group (Fig. 1A). CopB has eight probable transmembrane fragments, a large cytoplasmic loop between H6 and H7, and the characteristic His-N-MBD (Fig. 1C).

**A. fulgidus** CopB was cloned into a suitable expression vector and expressed in **E. coli**. A protein (T-CopB) lacking the first 54 amino acids of CopB was also generated to study the role of the N-His-MBD (Fig. 2). The presence of these two proteins in the corresponding **E. coli** membrane preparations used to characterize these enzymes. CopB-ATPase in the presence of activating metals (see below) showed temperature, ionic strength, and pH dependence similar to **A. fulgidus** CopA, a thermophilic Cu²⁺/Ag⁺-ATPase characterized previously (8). Thus, CopB showed maximum activity at pH 5.7, high ionic strength (400 mM NaCl), and at 70 °C–90 °C (Eₗ = 60.4 KJ/mol) (data not shown). Under these conditions where **E. coli** membrane ATPases are thermally inactivated, the background ATPase activity (in the absence of metals) was under 5%. Consequently, the functional characterization of CopB and T-CopB was feasible using **E. coli** membrane preparations.

**Metal Selectivity of CopB**—We hypothesized that CopB, as well as other homologous proteins having the Cys-Pro-His consensus in H6, would probably be activated by metals that are different from those transported by enzymes containing the Cys-Pro-Cys sequence in H6 (Cu²⁺/Ag⁺ or Zn²⁺/Cd²⁺/Pb²⁺). To test this possibility, the activation of CopB by different metals was measured. Fig. 3 shows that CopB is activated mainly by Cu²⁺ and, to a lesser extent, Ag⁺ (55%) and Cu⁺ (25%). A small but detectable activity was observed in the presence of Cd²⁺, Ni²⁺, and Zn²⁺. No further activation was observed at higher concentrations (up to 100 μM) of the tested metals. To verify the specificity of this activation and the absence of misleading artifacts, the effect of bicinechonic acid (BCA) on the CopB activation was tested. As expected, BCA did not affect Cu²⁺, Ni²⁺, or Cu⁺ activation of CopB but removed all of the Cu⁺-driven activity (Fig. 3). We have previously shown that **A. fulgidus** Cu²⁺-ATPase CopA is activated by the presence of Cu⁺ at millimolar concentrations (8). The stimulatory effect of Cu⁺ was not observed when CopB was activated by either Cu²⁺ or Cu⁺ (Fig. 3).

**Cu²⁺ Transport by CopB**—Because ATPase activity determinations show that CopB is largely activated by Cu²⁺, it was pertinent to verify that the divalent metal was transported by this enzyme. For these experiments, everted vesicles were prepared from CopB-expressing **E. coli** and ATP-dependent uptake was measured at 55°C. At this temperature, the enzyme had a significant easily measurable activity while minimal disruption of the vesicles integrity was observed. Fig. 4 shows that while vesicles from untransformed cells showed no ATP-dependent transport, as expected from ATPase activity determinations, CopB was able to transport Cu²⁺. Thus, the data presented in Figs. 3 and 4 support the concept that enzymes in the P₁₈₃₉ subgroup are Cu²⁺-transporting ATPases.

**Cu²⁺ Dependence of CopB-ATPase Activity**—To further explore the interaction of metal with CopB, the dependence of CopB-ATPase activity on Cu²⁺ and Cu⁺ was tested (Fig. 5). Cu²⁺ activates CopB with a high apparent affinity. Surprisingly, Cu⁺ activates the enzyme with an only slightly higher Kₐ for this transition. Copper in both redox states was inhibitory at high concentrations. This might be attributed to the metals rebinding with low affinity the E₂ form of the enzyme, i.e. the outward facing metal bind-

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**FIG. 2. Expression of CopB and T-CopB from A. fulgidus in E. coli.** Membrane fractions from untransformed E. coli (lanes 1 and 4) and membrane fractions of arabinose-induced E. coli transformed with pBAD/TOPO-CopB (lanes 2 and 5) or pBAD/TOPO-T-CopB (lanes 3 and 6) are shown. Lanes 1–3, Coomassie Brilliant Blue-stained gel; lanes 4–6, blot immunostained with anti-(His)₆-kerosidase peroxidase antibodies.

**FIG. 3. Activation of CopB-ATPase by metals.** The ATPase activity was determined as indicated under “Experimental Procedures.” Final concentration of each tested metal ion was 1 μM, which is a saturating concentration for all of them. 2.5 mM DTT was included in Cu⁺-containing assay mixture. Bars indicate activity in the presence of each metal (white) upon the addition of BCA (gray) or 20 mM cysteine (striped). 100% = 5.0 μmol/mg/h.
ing sites. This would slow the forward step of the catalytic cycle (E\textsubscript{2} \rightarrow E\textsubscript{1}) and consequently decrease the enzyme turnover rate as observed in other P-type ATPases (42). We have previously observed that activating metals drive CopA turnover at different rates, Ag\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} \text{faster than Cu}^2\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} (8). This is associated with the faster release of Ag\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} from the bound metal form of the enzyme (E\textsubscript{1}P(Ag)). Differences in the rate of metal release could be observed by monitoring the fast dephosphorylation of the E\textsubscript{2}P form that follows metal release (E\textsubscript{2}P \rightarrow E\textsubscript{2}P + Ag\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f}). Expecting a phenomenon similar to that seen for CopA, the kinetics of CopB phosphorylation and dephosphorylation reactions in the presence of either Cu\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{f} or Cu\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} were measured. In preliminary experiments, the dependence of CopB phosphorylation on ATP, Cu\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{f}, and Cu\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} was characterized. It was observed that maximum phosphorylation was achieved at 5 \mu M ATP (K\textsubscript{m} = 0.7 \pm 0.2 \mu M) and 1–10 \mu M metal (data not shown). Fig. 6A shows that CopB is phosphorylated at a significantly slower rate in the presence of Cu\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} compared with that in the presence of Cu\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{f}. This differences might be associated with different phenomena where the E\textsubscript{1} + metal \leftrightarrow E\textsubscript{1}P(meal) or the ATP-E\textsubscript{1}P(meal) \rightarrow E\textsubscript{1}P(meal) + ADP equilibrium are displaced to the left in the case of Cu\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f}. On the other hand, the E\textsubscript{2}P dephosphorylation rate was not significantly affected by the metal present, suggesting that either Cu\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} and Cu\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{f} release rates from the E\textsubscript{1}P(meal) form are roughly similar or alternatively that in the case of CopB the E\textsubscript{2}P \rightarrow E\textsubscript{2}P + P is slower than E\textsubscript{1}P(meal) \rightarrow E\textsubscript{2}P + metal (Fig. 6B). Aside from these possibilities, it is apparent that the different rate of phosphorylation in the presence of Cu\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{f} or Cu\textsuperscript{+}/H\textsubscript{11001}\textsuperscript{f} determines the observed variations in enzyme turnover driven by the alternative redox forms of the metal.

**Functional Role of the His-N-MBD**—The role of the His-N-
concentration of each metal ion was 1 μM. 2.5 mM DTT was included in Cu²⁺-containing assay mixture. Bars indicate activity of CopB (white) and T-CopB (gray) in the presence of the indicated metal. Inset, relative expression levels of CopB and T-CopB. Immunoblot a serial dilution of a mixture of equal amounts of CopB and T-CopB membrane preparations used in these assays.

MBD in the CopB catalytic cycle was studied by characterizing a truncated form of the enzyme, T-CopB. Removal of His-N-MBD leads to a significant reduction (50–60%) of the enzyme ATPase activity (Fig. 7). However, despite its lower activity, T-CopB showed a pattern of activation by various metals similar to CopB. It is relevant to point out that the levels of CopB and T-CopB in the corresponding membrane preparations from E. coli were similar (Fig. 7, insets). Thus, the lower activity of T-CopB was not associated with a lower expression level of the truncated protein. To understand the mechanism by which the His-N-MBD controls the enzyme turnover, the ligand dependence of T-CopB activity was examined. Fig. 8 shows that removal of this domain did not affect the activation of the enzyme by Cu²⁺. This suggests that the His-N-MBD has no role in affecting the binding of metal to the transport site located in the transmembrane region. Similarly, removal of the His-N-MBD did not modify the enzyme activation by ATP acting with low affinity (Fig. 9). Because the enzyme apparent affinity for ATP (measured under turnover conditions) is dependent on the E₄ ↔ E₃ equilibrium, the observed results indicate that His-N-MBD does not affect the enzyme distribution between these two forms. Different N-terminal MBDs characterized by the CXXC sequence are present in other P₁B-type ATPase subgroups (6, 19, 20). Studies of these domains have shown that they regulate the enzyme activity by controlling the rate of metal release/enzyme dephosphorylation of the E₄P(metal) form. To explore the possibility that His-N-MBD might affect the enzyme by a similar mechanism, the dephosphorylation of T-CopB was examined. Interestingly, although no differences were observed in the phosphoenzyme levels of T-CopB compared with CopB, a slower dephosphorylation rate was observed for the truncated enzyme (Fig. 10). This finding suggests that the His-N-MBD would influence the rate of metal transport by affecting the metal release/enzyme dephosphorylation steps in the CopB catalytic cycle.

**DISCUSSION**

P₁B-type ATPases play fundamental roles in maintaining metal homeostasis in diverse organisms (4–6). However, to fully describe their function, it is relevant to establish their particular metal specificity and understand the structural and mechanistic determinants of metal selectivity. This is complicated by the array of metal specificities that they present. On one hand, diverse P₁B-type ATPases transport different metals. For instance, Cu²⁺-ATPases, Zn²⁺-ATPases, a Co²⁺-ATPase, and in this study a Cu²⁺-ATPase have been reported (4, 5). On the other hand, a given enzyme can transport various metals at different rates and with distinct affinities (Cu²⁺/Ag⁺-ATPases (7, 8), Zn²⁺/Cd²⁺/Pb²⁺-ATPases (10–12)). We recently reported the identification of conserved residues in the transmembrane region of P₁B-type ATPases probably involved in metal coordination during transport. This analysis allowed us to sort these
enzymes into five subgroups, each with a singular metal specificity. For instance, Cu\(^{2+}\)/Ag\(^{+}\) (P\(_{1B-1}\)-subgroup) and Zn\(^{2+}\)/Cd\(^{2+}\)/Pb\(^{2+}\) (P\(_{1B-2}\)-subgroup) ATPases have a similar Cys-Pro-Cys sequence in their H6 but quite distinct sequences in H7 and H8. Thus, enzymes in both subgroups could be differentiated. P\(_{1B-1}\)-ATPases containing a Cys-Pro-His sequence in H6 and a His-N-MBD were grouped in the P\(_{1B-3}\)-subfamily. Although these enzymes have clearly different structural features, the only studied member of this group was previously characterized as a Cu\(^{2+}/Ag^{+}\)-ATPase functionally similar to those in P\(_{1B-1}\)-subgroup (14, 27). To establish the metal specificity of the P\(_{1B-3}\)-ATPases and their consequent role in metal homeostasis, we have investigated the functional characteristics of a member of this subgroup, CopB from A. fulgidus.

Functional Characteristics of A. fulgidus CopB—Our results show that CopB is a Cu\(^{2+}\)-ATPase that drives the efflux of Cu\(^{2+}\) from the cell. Its functional characterization indicates that it has a transport mechanism similar to that of other P-type ATPases, i.e. ion transport is coupled to the hydrolysis of ATP, an acid-stable intermediary is formed in the presence of ATP and the outwardly transported metal, and ATP interacts with the enzyme with two apparent affinities. Some of the enzyme parameters were slightly different from those previously reported for P\(_{1B}\)-ATPases. For instance, the CopB-ATPase K\(_{m}\) (0.7 \mu M) value is smaller than that reported for A. fulgidus CopA (4.8 \mu M) (8). On the other hand, the K\(_{V}\) of ATP for ATPase activation (83 \mu M) was similar to that of E. coli ZntA (75 \mu M) (29) but smaller than that observed in A. fulgidus CopA (250 \mu M) (8). It is interesting that CopB shows an apparent affinity for the transported metal (Cu\(^{2+}\), K\(_{V} = 0.28 \mu M\)) significantly higher than the metal affinities reported for CopA (Cu\(^{2+}\) (2.1 \mu M) and Ag\(^{+}\) (29 \mu M)) (8), Menkes protein (Cu\(^{2+}\), 2 \mu M) (30), or for ZntA (Zn\(^{2+}\) (11 \mu M), Cd\(^{2+}\) (5.5 \mu M), and Pb\(^{2+}\) (4.8 \mu M) (29). Thus, A. fulgidus CopB appears as the P\(_{1B}\)-ATPase with the highest reported affinity for metal.

The specificity of CopB for Cu\(^{2+}\) is evident by comparing its ATPase activity in the presence of various metals at saturating concentrations (Fig. 3). CopB is predominantly activated by Cu\(^{2+}\), although it is also partially activated by Cu\(^{+}\) and Ag\(^{+}\). Transport of these monovalent metals by E. hirae CopB has been described previously (14). The ATP-dependent uptake of \(^{64}\text{Cu}\)Cu\(^{2+}\) into sealed vesicles from CopB expressing E. coli further corroborates the ATPases activity results. Lastly, the preference of the enzyme for oxidized copper is also supported by the faster rate of enzyme phosphorylation in the presence of Cu\(^{2+}\) (Fig. 6A). Surprisingly, no differences were observed in the apparent affinity of CopB for Cu\(^{2+}\) and Cu\(^{+}\) as assessed by ATPase activation determinations (Fig. 5).

CopB has two putative metal binding regions, both characterized by the presence of His residues that might influence the enzyme specificity. The transmembrane metal binding site that likely coordinates the metal during transport probably determines the enzyme specificity. Although the His-N-MBD can probably bind several metal atoms, it does not seem to be involved in metal selectivity and actually is not essential for enzyme activity (Figs. 7 and 8). Within the transmembrane binding site, the His in H6 is probably the controlling element in Cu\(^{2+}\) transport in CopB. The idea is supported by our observation that the His in H6 and a Ser in H8 appear as the only difference among those transmembrane residues conserved in Cu\(^{2+}\)-ATPases (P\(_{1B-1}\) group) and Cu\(^{2+}\)-ATPases (P\(_{1B-3}\) group). The His, a Lewis base stronger than Cys, would facilitate the binding of Cu\(^{2+}\), a Lewis acid stronger than Cu\(^{+}\).

Functional Role of A. fulgidus CopB His-N-MBD—Our results show that the His-N-MBD is not essential for enzyme function but is required for maximum enzyme activity. Removal of the first 54 amino acids had little effect on the enzyme selectivity and did not affect the enzyme affinity for Cu\(^{2+}\) (Fig. 8) or its preference to remain either in E1 or E2 conformation (Fig. 9). However, the truncated protein showed a significantly reduced V\(_{\text{max}}\). This was correlated by a slower dephosphorylation rate observed for the truncated protein. A simple model might propose that the role of His-N-MBD is to regulate the rate of transport. It could be proposed that metal binding to the His-N-MBD promotes its interaction with one or more of the protein cytoplasmic loops. In turn, this interaction might drive a faster enzyme dephosphorylation. Further studies are necessary to test this hypothesis. Different from CopB and its homologous P\(_{1B-3}\)-ATPases, most P\(_{1B}\)-ATPases contain N-MBDs characterized by the CXXC metal binding sequence. Interestingly, mutation of these Cys residues lead to functional effects quite similar to those observed after removing His-N-MBD from CopB, suggesting an analogous role for both types of cytoplasmic metal binding domains.

An important characteristic of N-MBDs containing CXXC is their interaction with copper chaperones. Thus, the delivery of Cu\(^{+}\) to the CXXC N-MBD of Cu\(^{2+}\)-ATPases by chaperones has been shown (3, 32). A similar interaction of His-N-MBDs with chaperones is difficult to conceive taking into account the probable structural differences among the two subtypes of N-MBD. However, it might be considered that while the chaperone carries and maintains copper in its reduced state, CopB would transport Cu\(^{2+}\). This is the stable form of copper in the aqueous...
medium. Consequently, CopB might not need to interact with a chaperone to sense copper. In this direction, we have shown that in the absence of a chaperone, millimolars of Cys are required in the assay media for maximum activity of *A. fulgidus* CopA, a Cu$^{2+}$-ATPase (8). On the contrary, CopB does not require Cys for activity (Fig. 3).

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