Functional Roles of Metal Binding Domains of the *Archaeoglobus fulgidus* Cu^+-ATPase CopA†

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ABSTRACT: CopA, a thermophilic membrane ATPase from *Archaeoglobus fulgidus*, drives the outward movement of Cu^+ or Ag^+ [Mandal et al. (2002) *J. Biol. Chem. 277*, 7201−7208]. This, as other P_{IB}-ATPases, is characterized by a putative metal binding sequence ($C^{380}PC^{382}$) in its sixth transmembrane fragment and cytoplasmic metal binding sequences in its NH_2- and COOH-terminal ends ($C^{27}AMC^{30}$ and $C^{751}HHC^{754}$). Using isolated CopA, we have studied the functional role of these three putative metal binding domains. Replacement of transmembrane Cys residues by Ala results in nonfunctional enzymes that are unable to hydrolyze ATP. However, the CPC → APA substituted enzyme binds ATP, indicating its correct folding and suggesting that enzyme turnover is prevented by the lack of metal binding to the transmembrane site. Replacement of C-terminal Cys by Ala ($C^{751,754}A$) has no significant effect on ATPase activity, enzyme phosphorylation, apparent binding affinities of ligands, or E1−E2 equilibrium. In contrast, replacement of Cys in the N-terminal metal binding domain (N-MBD) ($C^{27,30}A$) leads to 40% reduction in enzyme turnover. The $C^{27,30}A$ enzyme binds Cu^+, Ag^+, and ATP with the same high apparent affinities as the wild-type CopA. Evidence that N-MBD disruption has no effect on the E1−E2 equilibrium is provided by the normal interaction of ATP acting with low affinity and the unaffected IC_{50} for vanadate inhibition observed in the $C^{27,30}A$-substituted enzyme. However, replacement $C^{27,30}A$ slowed the dephosphorylation of the E2P(metal) form of the enzyme, suggesting a reduction in the rate of metal release. Other investigators have shown the Cu-dependent interaction of isolated N-MBDs from the Wilson disease Cu-ATPase with the ATP binding cytoplasmic domain [Tsivkovskii et al. (2001) *J. Biol. Chem. 276*, 2234−2242]. Therefore, the data suggest a regulatory mechanism in which the Cu-dependent N-MBD/ATP binding domain interaction would accelerate cation release, the enzyme rate-limiting step, and consequently Cu^+ transport.

Archaeoglobus fulgidus CopA is a thermophilic membrane ATPase that selectively drives the outward transport of Cu^+ or Ag^+ (*1*). CopA belongs to the P_{IB} subfamily of P-type ATPases (*2*). P-type ATPases transport ions against their concentration gradient using the energy provided by ATP. Their catalytic cycle is characterized by the conformational transitions (E1 ↔ E2) and the formation of a phosphoester intermediate in their DKTGT consensus sequence (*2*−*5*). Enzymes in the P_{IB} subgroup transport heavy metals (Cu^+, Ag^+, Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}) (*2*, *6*). Found in archaea, prokaryotes, and eukaryotes, these are critical for transition metal homeostasis and in mechanisms of biotolerance (*6*−*8*). Their relevance is evident when considering that mutations of the two human Cu^+-ATPases are responsible for Menkes and Wilson diseases (*7*, *9*, *10*). Similarly, the abundance of CPx-ATPases in plants (eight genes in the *Arabidopsis thaliana* genome) speaks of fundamental and complex roles for these proteins in plant micronutrient metal metabolism (*11*−*13*).

Various structural−functional characteristics differentiate P_{IB}-ATPases from other P-type ATPases, most notably the presence of several metal binding domains (*2*, *5*−*8*, *10*, *14*). By similarity with other P-type ATPases, it can be assumed that metal binding sites involved in transport are located in the transmembrane region (*15*−*20*). The sixth transmembrane segment (H6) of CPx-ATPases is characterized by putative metal binding sequences (CPC, CPH, CPS, TPC, or SPC) that appear to be involved in determining enzyme specificity (Argüello, unpublished results). Experimental evidence supports the idea that these "CPx sequences" are required for enzyme function, probably participating in metal binding or transport. Mutation of CPC → CPA in the *Caenorhabditis elegans* Cu-ATPase yielded a protein unable to rescue a Cu-ATPase-deficient yeast mutant (Δ*ccc2*) (*21*). Similar results were observed when the CPH → SPH mutation was introduced in *Enterococcus hirae* CopB (*22*). Mutation of Cys in the CPC of *Escherichia coli* CopA, a Cu^+-ATPase, resulted in loss of copper resistance, transport, and phosphoenzyme formation (*23*). However, the participation of these residues on metal coordination has not been shown. Thus, it cannot be disregarded that the observed results might be associated with alternative effects of these replacements on the enzyme structure or interaction with other ligands.

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Many CPx-ATPases also have N-terminal metal binding domains (N-MBDs)[1] (*2*, *5*−*7*, *10*, *14*, *24*). These domains are approximately 60−70 amino acids long and contain the metal binding consensus sequences CXXC. Different, less frequent His-rich binding domains are present in other subtypes of CPx-ATPases (*25*). N-MBDs carrying the CXXC consensus sequence appear in five to six repeats in mammalian proteins. However, most CPx-ATPases have only two, one, or no N-MBD. The absence of N-MBDs in many of these proteins suggests a regulatory rather than an essential role for these domains in the catalytic mechanism of CPx-ATPases. N-MBDs are homologous to a number of metal chaperone proteins (*24*), can bind Cu^+, Cu^{2+}, Zn^{2+}, and Cd^{2+} (*26*−*29*), and exchange metals with the related chaperones through a simple equilibrium ($K_{eq} \approx 1$) (*30*). The NMR structure of the fourth N-MBD of the Menkes protein clearly points out the similarities between these domains and metal chaperones (*31*). Metal binding to the N-MBDs is required for targeting of Menkes and Wilson's proteins from the trans-Golgi network to the plasma membrane and a vesicular compartment, respectively (*32*−*36*). Studies directed to identify the effects of metal binding to N-MBDs on the enzyme structure and metal transport have postulated different roles depending on observed alterations in catalysis, transport, or particular partial reaction. Several reports have indicated that mutation of metal binding Cys or removal of N-MBDs (truncated proteins) leads to a reduced enzyme activity as determined by transport or ATPase assays (*37*−*39*). In the case of enzymes with several N-MBDs, such as the Menkes Cu-ATPase, mutations of different subsets of these lead to either decrease or no change in the enzyme V_{max} (*37*, *40*). In other cases, depending on the experimental system, the decrease in activity has been associated with a reduction (*40*), increase (*39*), or no change (*23*, *37*, *38*) in metal affinity. Phosphorylation experiments have suggested that mutation of all six Menkes protein N-MBDs renders an enzyme with an apparent tendency to favor a vanadate-sensitive E2 conformation. Concerning nucleotide binding, replacement of Cys in the three N-terminal N-MBDs of Menkes Cu-ATPase does not affect the low-affinity interaction of ATP with the enzyme (*40*). However, in a different experimental system, Lutsenko and collaborators have shown that the isolated N-MBDs from Wilson protein interact with the isolated cytoplasmic loop of the enzyme (*41*). The presence of Cu^+ decreases the interaction, and this leads to a reduction in the affinity of the isolated cytoplasmic loop for nucleotides. Thus, despite these contributions using different approaches, no consensus or integrated view has emerged on the molecular mechanism by which metal binding to N-MBDs affects CPx-ATPase activity.

We recently described the heterologous expression, solubilization, and purification of CopA, a thermophilic Cu^+-ATPase from *A. fulgidus* (*1*). We determined the kinetic parameters that define P-type ATPases: interaction of ATP with high and low affinity, maximum phosphorylation levels, turnover number, and inhibition by micromolar vanadate. We also recognized the enzyme specificity for Cu^+ and Ag^+, the only metals that drive CopA ATPase activity. By characterizing the distinct kinetics in the presence of Cu^+ or Ag^+, we explained that although CopA binds Cu^+ with 15 times higher apparent affinity, Ag^+ drives a faster turnover because the E2P(Ag) form undergoes a faster dephosphorylation than the E2P(Cu) form. We now present studies where we took advantage of this CopA preparation, site-directed mutagenesis, available assays, and kinetic analysis to characterize the role of the different metal binding domains in the catalytic cycle of a CPx-ATPase. *A. fulgidus* CopA has a CPC metal binding sequence in its H6 ($C^{380}PC^{382}$) and two apparent cytoplasmic metal binding domains, in its NH_2- and COOH-terminal ends ($C^{27}AMC^{30}$ and $C^{751}HHC^{754}$) (Figure 1). Replacement of transmembrane Cys removes enzyme activity and phosphoenzyme formation by ATP in the presence of metals. However, it does not produce significant changes in the enzyme structure and allows the binding of nucleotides. These data suggest a role of transmembrane Cys in the catalytic metal binding. The study of CopA cytoplasmic MBDs indicates that the N-MBD regulates enzyme turnover while the C-MBD does not appear to have a functional role. Most importantly, different from previous reports, our data reveal that the N-MBD regulates enzyme function not by affecting ligand interaction or E1 ↔ E2 equilibrium but by modifying the rate of enzyme dephosphorylation.

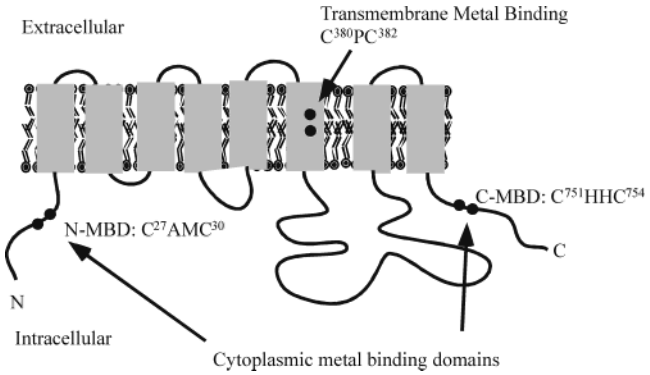

FIGURE 1: Schematic representation of the membrane topology and location of putative metal binding domains in *A. fulgidus* CopA.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Protein Expression. *A. fulgidus* CopA cDNA cloned into the pCRT7/NT-TOPO/His vector (Invitrogen, Carlsbad, CA) was used as template. Site-directed mutagenesis was performed using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were designed to introduce the following modifications: $C^{380}A$, $C^{382}A$, $C^{382}S$, $C^{380,382}A$, $C^{27,30}A$, $C^{751,754}A$, and $C^{27,30,751,754}A$. Mutations were confirmed by DNA sequencing. BL21Star(DE3)pLysS *E. coli* cells (Invitrogen, Carlsbad, CA) carrying the vector pSJS1240 encoding for rare tRNAs ($tRNA^{arg}AGA/AGG$ and $tRNA^{ile}$-AUA) (*42*) were used for expressing the substituted proteins after induction with 0.75 mM isopropyl β-D-thiogalactopyranoside.

Enzyme Preparation. Enzyme was prepared as described before (*1*). Briefly, membranes were isolated from cells expressing the wild-type (WT) CopA or the indicated mutants and treated with 0.75% dodecyl β-D-maltoside (DDM). The solubilized proteins were isolated by affinity chromatography using a Ni^{2+}-nitrilotriacetic acid column. The proteins were

[1] Abbreviations: DDM, dodecyl β-D-maltoside; DTT, dithiothreitol; N-MBD, N-terminal metal binding domain; P_i, inorganic phosphate; WT, wild type.

finally stored in 25 mM Tris, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% DDM, and 1 mM dithiothreitol (DTT) at −80 °C.

Functional Assays. Metal-dependent ATPase and phosphorylation assays were performed as described earlier (*1*). The time course of enzyme dephosphorylation was examined in samples phosphorylated in a medium containing 50 mM Tris, pH 7.5 (20 °C), 1 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 100 μM $AgNO_3$, 0.04 mM EGTA, 20 mM cysteine, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 20% dimethyl sulfoxide, and 0.05 mg/mL purified enzyme. After 30 s, ice-chilled 5 mM (final concentration) EDTA was added to the medium, and the samples were further incubated for 3 and 6 s at 0 °C.

Limited Proteolysis of WT and Mutant CopA. Trypsin treatment of WT and mutated enzymes was performed as described by Argüello and Kaplan (*43*). Briefly, protein (0.75 mg/mL) was treated with the enzyme:trypsin ratio indicated in the figures in a medium containing 20 mM Tris, pH 7.5, 0.01% asolectin, 0.01% DDM, and 3 mM $MgCl_2$ and in the presence of 3 mM ATP or 3 mM AMP. After 20 min at 37 °C, proteolysis was stopped by the addition of 1 volume of electrophoresis sample buffer. SDS−PAGE was carried out according to Laemmli (*44*) in 10% acrylamide gels. Protein bands were observed by staining the gels with Coomassie Brilliant Blue.

Data Analysis. Curves of ATPase activity vs Ag^+, Cu^+, or ATP concentrations were fit to $v = V_{max}L/(L + K_{1/2})$, where L is the concentration of the variable ligand. ATPase activity vs vanadate curves were fit to $v = (V_{max} - V_{min})/[1 + (I/K_{1/2})] + V_{min}$, where I is the concentration of inhibitor, $K_{1/2}$ is the inhibitor concentration that produces half the inhibitory effect, and V_{min} is the activity at maximum inhibition. The reported standard errors for V_{max}, K_m, and $K_{1/2}$ are asymptotic standard errors reported by the fitting program.

RESULTS

Effect of Cys Replacement in Putative Metal Binding Domains on CopA ATPase Activity. CopA has three putative metal binding domains (Figure 1). The transmembrane metal binding domain ($C^{380}PC^{382}$) and the N-MBD ($C^{27}AMC^{30}$) are similar to those observed in most CPx-ATPases. The putative metal binding sequence $C^{751}HHC^{754}$ in the C-terminus of the protein is unique to *A. fulgidus* CopA. To explore the role of these Cys in the catalytic function of CopA, they were replaced by site-directed mutagenesis. Proteins carrying single $C^{380}A$, $C^{382}A$, and $C^{382}S$ and double $C^{380,382}A$ replacements were designed to study the transmembrane binding site. Substitutions $C^{27,30}A$, $C^{751,754}A$, and $C^{27,30,751,754}A$ were engineered to characterize the cytoplasmic sites while ensuring their metal binding capacity was removed. These proteins were expressed in *E. coli*, solubilized, and purified (95% pure as indicated by Coomassie Brilliant Blue staining of the SDS−PAGE gel).

Figure 2 shows that enzymes carrying replacements of transmembrane Cys were inactive even in the presence of 100 μM metal, 50 times over the Cu^+ $K_{1/2}$ and 4 times over the Ag^+ $K_{1/2}$ observed for WT CopA (*1*) (see also Figure 4). This is in agreement with previous studies reporting the lack of transport capability in enzymes carrying similar mutations

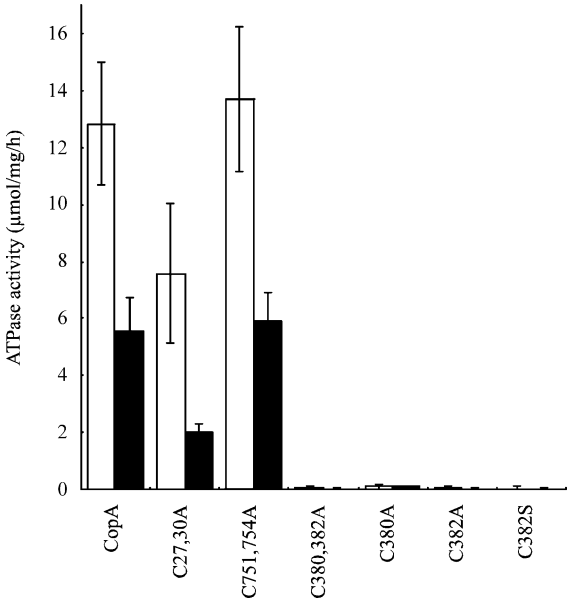

FIGURE 2: Effect of replacement of Cys at N-MBD, C-MBD, and CPC sites on CopA ATPase activity. Assays were performed at saturating metal concentrations, 100 μM Ag^+ (white bars) or Cu^+ (black bars). 2.5 mM DTT was included in the Cu(I)-containing assay mixture. Bars are the mean ± SE of three experiments performed with independent enzyme preparations.

(*23*). Replacement of Cys in cytoplasmic metal binding sites had different effects on enzyme activity. While substitution $C^{751,754}A$ had no effect on maximum enzyme activity, replacement of Cys in the N-terminal site ($C^{27,30}A$) led to a significant reduction in the ATPase activity (60% of Cu^+ activated and 40% of Ag^+ activated) (Figure 2). The mutant lacking both cytoplasmic metal binding sites ($C^{27,30,751,754}A$) had a reduction in activity similar to that of the $C^{27,30}A$ mutant (not shown).

Functionality of Proteins Carrying Substitutions in Transmembrane Cys. Transmembrane Cys appear to be essential for CopA ATPase activity. Enzymes carrying replacements in these Cys were unable to hydrolyze ATP and could not form a phosphorylated intermediary in the presence of ATP and metals (not shown). These effects might be due to the participation of these Cys in metal coordination or to unspecific structural alterations that prevent binding of ligands (other than metals) or required conformational transition. Consequently, it is relevant to verify that the substituted enzymes are properly folded and able to perform metal-independent ligand interaction or conformational transitions. In this direction, attempts to study metal-independent enzyme phosphorylation by inorganic phosphate (P_i) were unsuccessful, since conditions could not be established for significant back door phosphorylation of WT CopA. An alternative approach was then sought to obtain information on the status of the $C^{27,30}A$ mutant. The peptide pattern resulting from limited proteolysis of P-type ATPases is dependent on the enzyme conformation (*45*, *46*). This has proved to be a useful tool for the qualitative characterization of conformational transitions, interaction with ligand, and overall structural integrity. Using this methodology, the interactions of WT and $C^{380,382}A$ mutant CopA with the enzyme ligands were examined (Figure 3). Partial trypsin digestion of WT CopA in the absence of ligands yielded a particular pattern characterized by the presence of significant

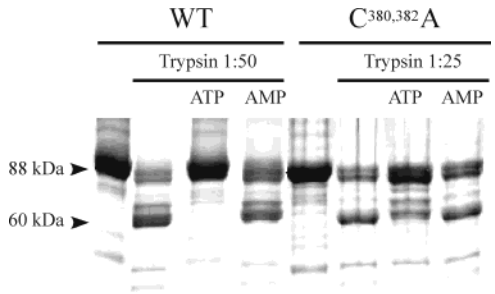

FIGURE 3: ATP-mediated protection of trypsin digestion of WT and $C^{380,382}A$-substituted CopA. SDS−PAGE of proteins treated with trypsin (1:50 trypsin:enzyme ratio for WT and 1:25 trypsin: enzyme for mutant) in the absence and in the presence of 3 mM ATP or 3 mM AMP for 20 min at 37 °C.

≈60 kDa bands. The presence of ATP in the media largely prevented enzyme digestion indicating the interaction of the enzyme with the nucleotide. As expected, digestion in the presence of AMP yielded a pattern analogous to that obtained in the absence of nucleotide. Although the $C^{380,382}A$ CopA was less susceptible to digestion, trypsin treatment of the mutated protein revealed the cleavage protection by ATP (but not AMP), suggesting that the replacement of $C^{380,382}A$ does not affect nucleotide binding to the enzyme. Trypsin treatment of WT and mutated enzymes in the presence of other ligands (vanadate, metals) produced patterns similar to those in the absence of ligand, and consequently, this approach did not report their interaction with CopA (data not shown). In summary, data from Figures 2 and 3 and phosphorylation experiments indicate that $C^{380,382}A$ CopA can interact with ATP but is unable to be phosphorylated, probably because the transmembrane metal transport site cannot bind metals.

Functional Role of Cytoplasmic Metal Binding Domains. The ATPase activity of $C^{27,30}A$-, $C^{751,754}A$-, and $C^{27,30,751,754}A$-substituted enzymes suggests that while the N-MBD (C^{27}-AMC^{30}) might play a regulatory role in the enzyme catalytic cycle, the putative C-MBD ($C^{751}HHC^{754}$) is not necessary for enzyme activity. Furthermore, the CopA C-terminal amino acid sequence aligns poorly with the well-characterized N-MBD of other CPx-ATPases or with Cu chaperones (not shown), suggesting that probably this is not a functional domain. Nevertheless, we further characterized the $C^{751,754}A$-substituted enzyme to discard other masked functional effects. For instance, Figure 4 shows that the $C^{751,754}A$ mutation had no effect on the enzyme apparent affinity for Cu^+. Similarly, no changes were observed in the ATP and Ag^+ dependence of ATPase activity, vanadate inhibition, or maximum phosphoenzyme levels (not shown). These data suggest that, independently of the metal binding capacity of the $C^{751}HHC^{754}$ sequence, this does not appear to influence the enzyme function.

Replacement $C^{27,30}A$ had a significant effect in the CopA ATPase activity (Figure 2). Since reduced V_{max} is observed at saturating concentrations of all substrates, this effect is not likely to be due to alterations in ligand binding. However, since previous reports have suggested that ligand interactions might be affected by metal binding to N-MBD (*37*−*40*), we investigated the dependence of the activity on metals and ATP. Figure 4 shows that the reduction in V_{max} is not associated with changes in the metal dependence since WT and $C^{27,30}A$ CopA show similar Cu^+ $K_{1/2}$ for ATPase

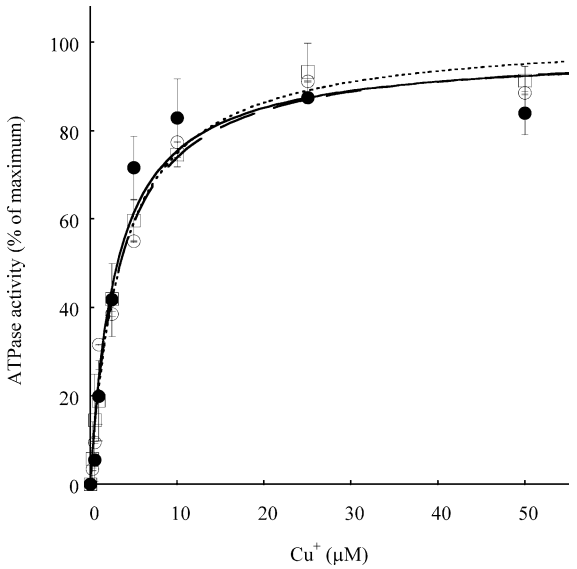

FIGURE 4: Cu^+ dependence of ATPase activity of WT and $C^{27,30}A$- and $C^{751,754}A$-substituted CopA. The ATPase activity was measured in the presence of the indicated Cu^+ concentrations and 2.5 mM DTT. Data were fitted using V_{max} = 100% and the following parameters: $K_{1/2} = 2.9 \pm 0.7$ μM for WT CopA (●), $K_{1/2} = 3.3 \pm 0.6$ for $C^{27,30}A$ (○), and $K_{1/2} = 3.6 \pm 0.3$ for $C^{751,754}A$ CopA (□). 100% activities were 10−11, 4−5, and 9−11 μmol mg^{-1} h^{-1} for WT, $C^{27,30}A$, and $C^{751,754}A$ CopA, respectively. Points are the mean ± SE of three experiments performed with independent enzyme preparations.

activation. Identical results were observed when Ag^+ activation was measured (not shown). Furthermore, since the apparent affinity for metals was not affected and the V_{max} observed in the presence of Cu^+ and Ag^+ was proportionally reduced (Figure 2), the participation of the N-MBD in the enzyme selectivity mechanism appears unlikely.

As in other P-type ATPases, ATP interacts with CopA with two apparent affinities, in the micromolar and submillimolar range (*1*). Taking into account the catalytic cycle of P-type ATPases, the high-affinity interaction would report on the status of the ATP binding site (*47*, *48*). In our studies, to evaluate the effect of the $C^{27,30}A$ mutation on the ATP binding site, the ATP dependence of phosphoenzyme formation under nonturnover conditions was measured. Figure 5 shows that the mutation did not affect the maximum phosphoenzyme level obtained at saturating ATP concentrations nor the ATP K_m for enzyme phosphorylation. This suggests that the mutation did not modify the ATP/enzyme interaction or the structure of the ATP binding site.

Removal of the metal binding capacity of the N-MBD does not seem to affect the interaction of the enzyme with metals at the transport/activation site or nucleotides at the phosphorylation site. To explore the possibility that the mutation might affect the main conformational transitions E1 ↔ E2 (*40*), the dependence of ATPase activity on ATP and the inhibition by vanadate under turnover conditions were studied. These two parameters appear to be good reporters of alterations of this equilibrium (*49*), since ATP acts with low affinity, accelerating the E2 → E1 transition, and vanadate binds the E2 form of the enzyme (*4*). Figure 6 shows that ATP activated WT and $C^{27,30}A$-mutated CopA in an identical manner ($K_m = 0.27 \pm 0.15$ mM for WT and 0.22 ± 0.07 mM for $C^{27,30}A$). Similarly, vanadate inhibited both enzymes to the same extent with indistinguishable IC_{50}

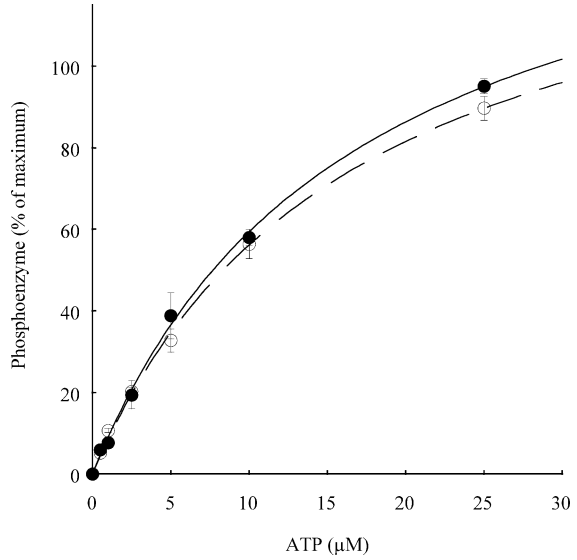

FIGURE 5: Phosphorylation by ATP of WT and $C^{27,30}A$-substituted CopA. The phosphoenzyme level of WT (●) and $C^{27,30}A$ (○) was measured at different concentrations of ATP as indicated. 100% phosphoenzyme levels were 0.7−0.9 nmol/mg. Data were fitted using maximum phosphoenzyme = 100% and the following parameters: $K_m = 16.6 \pm 1.5$ μM for WT CopA; $K_m = 16.5 \pm 1.3$ μM for $C^{27,30}A$-substituted CopA. Points are the mean ± SE of $n = 3$ experiments.

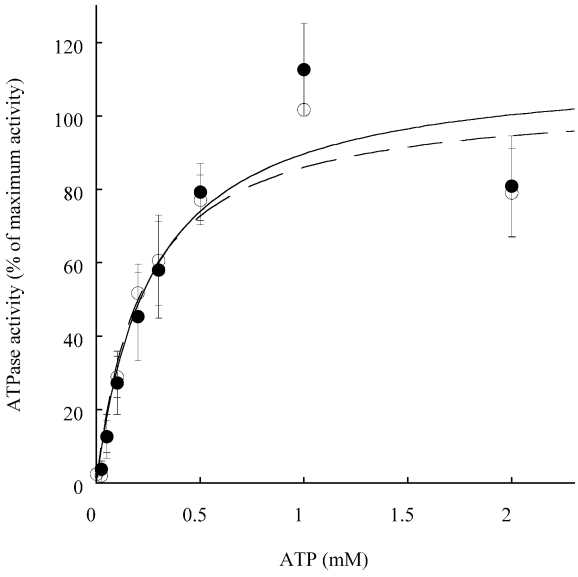

FIGURE 6: ATP dependence of ATPase activity of WT and $C^{27,30}A$-substituted CopA. ATPase activity was determined in the presence of 100 μM Ag^+ and ATP varied as indicated. Data were fitted using a constant V_{max} = 100% and an ATP K_m of 0.27 ± 0.1 mM for WT CopA (●) and 0.22 ± 0.07 for $C^{27,30}A$ CopA (○). 100% activities were 10−11 and 4−5 μmol mg^{-1} h^{-1} for WT CopA and $C^{27,30}A$ CopA, respectively. Points are the mean ± SE of three experiments performed with independent enzyme preparations.

(Figure 7). Thus, the data suggest that N-MBD does not affect enzyme function by modifying the E1 ↔ E2 equilibrium.

Reduction of an enzyme V_{max} observed under saturating substrate concentrations is likely associated with a decrease of the rate constant of the rate-limiting step. In previous studies of CopA, we observed that differences in the V_{max} generated by Cu^+ and Ag^+ could be linked to the distinct catalytic steps associated with metal release (*1*). This was followed by analyzing the subsequent forward dephosphorylation of the E2P form of the enzyme. Considering the effect of $C^{27,30}A$ replacement on CopA V_{max}, we studied the dephosphorylation kinetics of the mutated protein. $C^{27,30}A$-substituted and WT CopA enzymes were phosphorylated in the presence of ATP and Ag^+, and phosphoenzyme levels were measured after stopping phosphorylation by adding EDTA to the media to complex Mg^{2+}. Figure 8 shows a slower forward dephosphorylation for the $C^{27,30}A$-substituted

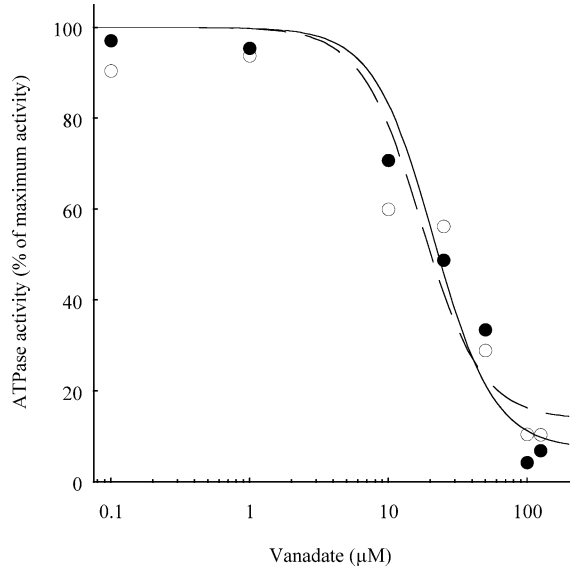

FIGURE 7: Vanadate inhibition of WT and $C^{27,30}A$-substituted CopA. ATPase activity of WT (●) and $C^{27,30}A$(○) CopA was determined in the presence of 100 μM Ag^+ and varied concentration of vanadate as indicated. Vanadate data were fitted using V_{max} = 100% and yielded the following parameters: $V_{min} = 1 \pm 3\%$, $K_{1/2} = 21.2 \pm 3.7$ μM for WT and $K_{1/2} = 17.3 \pm 4.6$ μM for $C^{27,30}A$. 100% activities were 10−11 and 4−5 μmol mg^{-1} h^{-1} for WT CopA and $C^{27,30}A$ CopA, respectively. Points are the mean of three experiments performed with independent enzyme preparations.

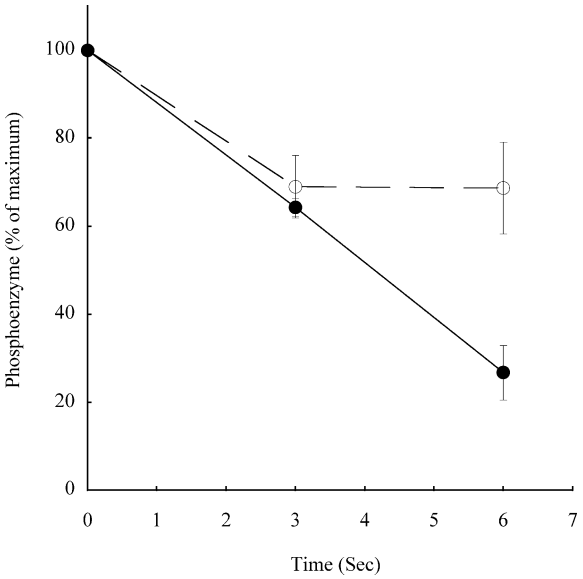

FIGURE 8: Dephosphorylation rate of WT and $C^{27,30}A$-mutated CopA. Phosphoenzyme levels were measured at different time intervals after addition of 5 mM EDTA to WT (●) and $C^{27,30}A$ (○) mutant enzymes phosphorylated for 30 s in the presence of 100 μM Ag^+ and 25 μM ATP. No curve fitting was attempted. 100% phosphoenzyme levels were 0.7−0.9 nmol/mg. Points are the mean ± SE of three experiments performed with independent enzyme preparations.

CopA. This significant reduction in the dephosphorylation rate might explain the observed effects on V_{max}. Most significantly, this finding supports a plausible regulatory mechanism in which, upon metal binding, N-MBD increases enzyme turnover by allowing a faster metal release and consequent enzyme dephosphorylation. An additional aspect of these data is the apparent biphasic nature of the dephosphorylation kinetics of the $C^{27,30}A$ mutant. A possible source of this behavior might be a probable dimer structure of solubilized CopA, as observed for other P-type ATPases (*50*, *51*).

DISCUSSION

CPx-ATPases are a subgroup of P-type ATPases responsible for the transport of heavy metals across biological membranes. The most intriguing aspect of these enzymes is their interaction with metals. For instance, while typical P-type ATPases (Ca-ATPase, Na,K-ATPase, etc.) have well-defined unique specificities for single cations transported in a given direction, CPx-ATPases have broader specificities, being able to transport two or three different metals. In this manner, some of them can transport Cu^+ or Ag^+, while others are activated by Zn^{2+}, Cd^{2+}, and Pb^{2+} (*1*, *23*, *52*, *53*). It can also be considered that they transport heavy metals, substrates that are not free in the intracellular milieu (*54*, *55*). The interaction of CPx-ATPases with metals is further complicated by the presence of more than one metal binding site in most enzymes of this subfamily. Despite this complexity, a detailed description of the metal−enzyme interactions is necessary for understanding CPx-ATPase physiological roles and mechanism of ion transport. The primary structure and membrane topology of CPx-ATPases suggest the presence of two types of metal binding sites: a site located in the transmembrane region and one or more sites in the cytoplasmic N-terminal end of most CPx-ATPases. Toward understanding these different metal binding sites, we have studied their role in a Cu^+-ATPase using as a model the thermophilic CopA from *A. fulgidus*. We recently developed expression and purification protocols that yield large amounts of a pure soluble form of this enzyme (*1*). This allows us to measure a number of functional variables under controlled conditions.

The Transmembrane Metal Binding Site of CPx-ATPases. As in most CPx-ATPases, in *A. fulgidus* CopA a putative transmembrane metal binding is defined, at least in part, by two Cys in H6 (C^{380} and C^{382}). Studies of homologous proteins using functional complementation and transport assays have indicated the essential role of these Cys for function (*21*−*23*). Although complementation assays do not provide quantitative information on the extent of functional alteration, transport measurements have shown that enzymes lacking one of the transmembrane Cys are unable to transport metals. Our data show that enzymes mutated at these positions have no ATPase activity and they are not phosphorylated by ATP in the presence of Cu^+ or Ag^+. Most importantly, these alterations do not appear to be associated with unspecific alterations in the enzyme structure. It is apparent that enzymes lacking metal binding capacity in the transmembrane region can still bind ATP and undergo conformational changes produced by nucleotide binding. Thus, although indirectly, the evidence suggests that enzymes mutated at Cys^{380} and Cys^{382} would not bind metal at the catalytic/transport site. A simple interpretation is that these Cys participate in metal coordination during transport. While this is probable, it does not imply that these are the only amino acids involved in this function. A minimal model of the transmembrane metal binding site should meet several requirements. It should explain the different metal selectivity observed in ATPases with the same CPC sequence in H6. For instance, specificity for Cu^+ (and Ag^+ in some cases) has been established for Menkes protein (*37*), Wilson proteins (*56*), *E. coli* CopA (*23*), and *A. fulgidus* CopA (*1*), while the selectivity for Zn^{2+} (and divalent metals Cd^{2+} and Pb^{2+}) has been observed in *E. coli* ZntA (*52*, *53*), *Staphylococcus aureus* (p1258) CadA (*57*), *Listeria monocytogenes* CadA (*39*), and *Bacillus subtilis* CadA (*58*). All of these proteins carry the same CPC sequence in H6. Thus, it is logical to assume that amino acids likely located in other transmembrane segments also participate in metal coordination at the transport sites. From a different perspective, it can be considered that the metal transport site binds cytoplasmic metals with high affinity and then through a conformational change opens to the extracellular media (or organelle) while decreasing its affinity for the metal. This can be better accomplished by coordination through amino acid side chains in different transmembrane segments, as is the case in the well-characterized Ca- and Na,K-ATPases (*15*−*20*).

The Cytoplasmic Metal Binding Sites of CPx-ATPases. A singular characteristic of CPx-ATPases is that many of them have additional metal binding domains located in their N-terminal cytoplasmic end, N-MBDs. Although it is apparent that there are different types of N-MBDs, the most frequent ones are characterized by a CXXC sequence. Diverse studies have explored the role of N-MDBs on CPx-ATPase (*23*, *24*, *26*−*41*). Studies of Menkes and Wilson proteins using functional complementation indicate that results (cell survival) depend on the number and position of altered N-MBDs and the type of modification (mutation or truncation). Studies on the molecular effects of mutations at N-MBDs have shown reductions in V_{max} depending on the system under study and the extent of modification of N-MBDs (*23*, *37*, *38*, *40*). Alterations in metal and nucleotide affinities have been reported in addition to the reduction of V_{max} (*23*, *37*−*40*). However, modification of these parameters would not be the cause of the observed decrease in V_{max}, since this is measured at saturating ligand concentrations.

We have used a simple protein, CopA, with a single functionally relevant MDB to examine its role in enzyme function. The highly purified preparations of this enzyme (and mutants under study) allow us to measure enzyme activity and to analyze different ligand interactions with the protein, the E1 ↔ E2 equilibrium, and the kinetics of some partial reactions. We assumed that replacement $C^{27,30}A$ removes the metal binding capacity from CopA N-MBD and measured the effect of this modification on enzyme function. We observed that lack of metal binding to the N-MBD had no effect on the metal activation of CopA. This reveals important mechanistic aspects. First, the metal binding at cytoplasmic sites does not affect binding at the transport sites and, of course, does not participate in transmembrane transport. Second, the independent metal binding to transport sites also dispels the possibility that the transported ion would be "delivered" through the N-MBD into the transmembrane transport path in a two-step process.

We also explored the possibility that the N-MBD might regulate the interaction of the enzyme with ATP or the distribution of enzyme between the E1 and E2 states during turnover (*40*, *41*). Our data show that CopA binds ATP with high affinity independently of metal binding to the N-MBD and, similarly, metal binding to N-MBD does not significantly affect the equilibrium among major enzyme conformations.

An enzyme V_{max} is primarily determined by the rate-limiting step of its catalytic cycle. In previous studies, we observed that metal release from their transport site appears to be the rate-limiting step in CopA (*1*). This is evident from the different dephosphorylation rates observed for E2P(Ag) and E2P(Cu). In turn, this leads to the different V_{max} observed in the presence of these two metals (Ag^+ or Cu^+). Keeping this in mind, we analyzed the effect of the $C^{27,30}A$ mutation on the enzyme dephosphorylation rate. As expected, the mutated enzyme dephosphorylated at a slower rate which explains the observed reduced V_{max}. Our findings point out a regulatory mechanism in which metal binding to the cytoplasmic N-MBD influences the rate of metal release and consequently the rate of transport. Clearly, this model requires conformational changes in the N-MBD upon metal binding that lead to structural modifications in the transmembrane metal binding domain. Interestingly, Lutsenko and co-workers showed that the Wilson N-MBDs interact with the enzyme's large cytoplasmic loop in a Cu^+-dependent manner (*41*). The structural−functional linkage between the ATP binding and the metal transport sites, via transmembrane helixes extending into the cytoplasmic domain, has been shown for other P-type ATPases (*18*, *59*, *60*). It is then possible to postulate that the metal binding to N-MBDs reduces its interaction with the ATP binding domain, this structural modification is communicated to the metal transport site via transmembrane segments, and this leads to a faster metal release and enzyme turnover.

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