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Mechanism of Metal delivery and binding to transport sites of Cu⁺-transporting ATPases

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**Mechanism of Metal delivery and binding to transport sites of
Cu⁺-transporting ATPases**

by

Ying Yang

A Thesis

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APPROVED:

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ABSTRACT

CopA, a thermophilic membrane ATPase from *Archaeoglobus fulgidus*, drives the outward movement of Cu^+ across cellular membranes. CopA contains at least two metal binding domains, a regulatory N-terminal Metal Binding Domain (N-MBD) and an occlusion/coordinating metal binding site in the 6th, 7th and 8th transmembrane segments. Previous studies showed that the presence of millimolar concentration of Cys is essential for CopA activity. The high affinity of CopA for metal in the presence of millimolar concentration of Cys (consider Cu-Cys $K_D < 10^{-10}$ M) suggests a multifaceted interaction of the enzyme with Cys. To elucidate the role of Cys, we studied its effect on the partial reactions of the catalytic cycle of CopA. We observed that 2-50 mM Cys accelerates enzyme turnover with little effect on the Cu^+ affinity of CopA. Cys accelerates enzyme phosphorylation, but has no effect on the dephosphorylation rates. Thus, Cys increases steady state phosphoenzyme levels. Besides, Cys has no significant effect on $E_1 \leftrightarrow E_2$ equilibrium. Similar results were observed in truncated CopA lacking the N-MBD suggesting that enzyme activation by Cys is independent of the regulatory metal binding sites. These results and the kinetic analysis of activation curves suggest that while Cu^+ is delivered to the transport site as a Cu-Cys complex, Cys in the mM range stimulates the ATPase acting as a non-essential activator.

Key words: P_{IB} -type ATPase, CopA, Cys, Cu^+ , heavy metal binding, metal transport.

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LIST OF ABBREVIATIONS

MNK: Menkes protein

WND: Wilson protein

SR: Sarcoplasmic reticulum

TM: Transmembrane segment

DTT: Dithiothreitol

IC₅₀: Concentration of inhibitor required for a 50% decrease in enzyme activity

N-MBD: N-terminal metal binding domain

P_i: Inorganic phosphate

SOD: Super oxide dismutase

WT-CopA: Wild type CopA

T-CopA: Truncated CopA lacking N-MBD

IPTG: isopropyl β-D-thiogalactopyranoside

Ni-NTA: Ni²⁺-nitrilotriacetic acid

PMSF: Phenyl methyl sulfonyl fluoride

DDM: dodecyl-β-D-maltoside

GSH: Glutathione

1. INTRODUCTION

1.1. *Cu Homeostasis*

Heavy metal ions such as Fe, Cu, Zn and Mn play many critical roles in cells (Pena et al. 1999). Among them, Cu is an essential trace element with key physiological and biochemical functions. There are numerous Cu-containing proteins that are involved in a diverse range of biological processes including, oxidative phosphorylation (cytochrome-*c* oxidase), cellular antioxidant activity (superoxide dismutase), connective tissue formation (lysyl oxidase) and iron metabolism (ceruloplasmin) (Linder and Goode 1991; Vulpe and Packman 1995; Pena et al. 1999). The ability of Cu to cycle between a stably oxidized-Cu²⁺-form and unstable reduced-Cu⁺-form states is exploited by cuproenzymes involved in redox reactions, e.g. Cu/Zn superoxide dismutase and cytochrome oxidase. While trace amounts of Cu is needed to sustain life, excess amount of Cu is extremely toxic (Glynn 1985). The Cu²⁺ ↔ Cu⁺ transitions can generate reactive oxygen species, such as superoxide radical and hydroxyl radical. Most, if not all, cells have mechanisms for Cu homeostasis. In eukaryotes, Cu is taken into the cell by specific uptake systems and transferred to cytosolic chaperone proteins that deliver it to Cu-requiring enzymes, with little free cytosolic Cu ions (Vulpe and Packman 1995; Culotta et al. 1999). In fact, sophisticated homeostatic mechanisms together with efficient chelating agents and chaperones keep the free copper ions below 0.2 pM (O'Halloran and Culotta 2000). Cu is exported from the cells via Cu specific P_{1B}-type ATPases.

1.2. Cu Chaperone

Small cytosolic proteins called chaperones carry out Cu delivery to membrane-bound proteins, such as Cu-transporting ATPases, and to Cu-dependent enzymes. The Cu chaperones bind and deliver Cu ions to intracellular compartments and insert Cu into the active sites of specific partners, Cu-dependent enzymes.

Some Cu chaperones such as Atx1, Atox1, CopZ, and CCH bind Cu^+ and specifically interacts directly with the copper-binding domains of a P-type ATPase copper transporter. For instance, in humans, Atox1 is the Cu-chaperone for Menkes (MNK) and Wilson (WND) diseases proteins (Klomp et al. 1997; Hung et al. 1998; Walker et al. 2002). The 1.8Å structure of Atox1 revealed a Cu^+ ion coordinated by Cys residues from two adjacent Atox1 molecules. It suggested that direct metal ion exchange between MT/HCXXC containing domains could possibly be accomplished (Wernimont et al. 2000). In yeast, high affinity Cu-transporting proteins like Ctr1 and Ctr3 uptake Cu from outside of the cell. ATX1 delivers Cu^+ to Ccc2, one of the yeast homologues of MNK and WND (Solioz and Vulpe 1996; Lin et al. 1997; Huffman and O'Halloran 1999). Homologues of ATX1 have also been found in bacteria. For instance, CopZ of *Enterococcus hirae*, delivers Cu^+ to a responsive repressor, CopY, encoded by the same operon as CopZ (Cobine et al. 1999). In plant, CCH, a Cu^+ chaperone of Arabidopsis that is homologous to ATX1 was found to be upregulated in senescent leaves (Himmelblau et al. 1998).

COX17, COX15, COX11 and SCO1 are involved in Cu^+ shuttling to mitochondrial

membrane and incorporation of Cu^+ into cytochrome *c* oxidase (Glerum et al. 1997; O'Halloran and Culotta 2000). CCS delivers Cu^+ to Cu,Zn-superoxide dismutase (Culotta et al. 1999; Schmidt et al. 1999; Lamb et al. 2000).

1.3. P-type ATPases

P-type ATPases are ion pumps that transport a variety of ions (H^+ , Na^+ , K^+ , Cu^+ , Ca^{2+} , Mg^{2+} , Cd^{2+} , Cu^{2+} , Zn^{2+} , etc.) across cell membranes using the energy provided by ATP hydrolysis (Lutsenko and Kaplan 1995; Axelsen and Palmgren 1998; Kuhlbrandt 2004). The main structural characteristics of these enzymes are six to ten transmembrane α -helices (H1-H10), one ATP binding domain, and the presence of a highly conserved sequence (DKTGT) in the large cytoplasmic loop. The phosphorylation of the conserved Asp residue in this sequence drives the key conformational changes in the protein (Lutsenko and Kaplan 1995; Axelsen and Palmgren 1998; Kuhlbrandt 2004).

P-type ATPases constitute a large, ubiquitous and varied family of membrane proteins. This is divided into five branches based on sequence alignments and putative ion specificity (Lutsenko and Kaplan 1995; Axelsen and Palmgren 1998; Argüello 2003). The P_I group is divided into: a: bacterial Kdp-like ATPases and b: heavy metals (Cu^+ , Ag^+ , Cu^{2+} , Cd^{2+} , Zn^{2+} , Pb^{2+} , Co^{2+}) transporters. The P_{II} group includes: sarcoplasmic reticulum (SR) Ca^{2+} -ATPases, plasma membrane Ca^{2+} -ATPases, Na^+/K^+ and H^+/K^+ -ATPases. Group P_{III} : H^+ and Mg^{2+} transporters. Group P_{IV} are lipid transporters. P_V is a group with unknown substrate specificity.

Crystal structures at atomic resolution are available only for the SR Ca^{2+} -ATPase (Toyoshima et al. 2000; Toyoshima and Nomura 2002). The first high-resolution crystal structure of the SR Ca^{2+} -ATPase, determined at 2.6 Å resolution, depicted the protein in its E_1 conformation with two Ca^{2+} ions bound. The ion-binding sites are surrounded by the TM 4-6. The structure of SR Ca-ATPase in E_2 conformation, stabilized by its specific inhibitor thapsigargin, has also become available at a resolution of 3.1 Å (Toyoshima and Nomura 2002). These crystal structures indicate that the SR Ca^{2+} -ATPase consists of four well-defined protein domains (Fig. 1). According to their function or position, they are referred to as the Phosphorylation (P)-domain, the Nucleotide binding (N)-domain, the actuator (A)-domain and the membrane (M)-domain. Detailed comparison of the structures in the Ca^{2+} bound and unbound forms reveals that very large rearrangements of transmembrane helices take place accompanying Ca^{2+} dissociation-binding and that they are mechanically linked with equally large movements of the cytoplasmic domains. The most surprising discovery was the long distance between the nucleotide-binding and the phosphorylation sites (25 Å), and the even longer distance between the phosphorylation and the ion-binding sites in the membrane (~45 Å).

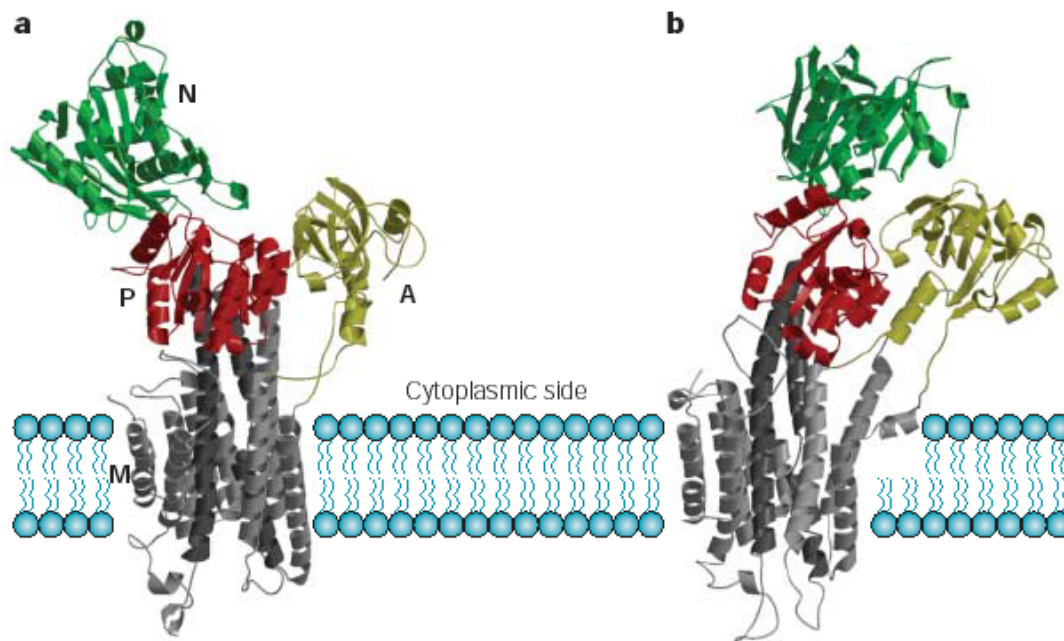


Fig. 1. SR Ca²⁺-ATPase structures and models. *A.* Ca²⁺-ATPase in the E₁ state (Toyoshima et al. 2000). *B.* SR Ca²⁺-ATPase in the thapsigargin-inhibited E₂ state (Toyoshima and Nomura 2002). The phosphorylation (P)-domain is shown in *red*, the nucleotide binding (N)-domain in *green*, the actuator (A)-domain in *yellow*, the membrane (M)-domain in *grey*.

1.4. *P_{1B}*-type ATPases

P_{1B}-type ATPases have been found in bacteria (Rogers et al. 1991; Okkeri and Haltia 1999; Rensing et al. 2000), archaea (Mandal et al. 2002), yeast (Rad et al. 1994; Catty et al. 1997), plants (Tabata et al. 1997; Thomine et al. 2000; Eren and Arguello 2004; Gravot et al. 2004) and animals (Bull et al. 1993; Vulpe et al. 1993; Wu et al. 1993; Lutsenko and Petris 2003). Mutations in human Cu⁺-efflux pumps cause the rare, but lethal Menkes and Wilson diseases. Because of their medical importance, these pumps have received much attention since their discovery more than ten years ago (Bull et al. 1993; Silver et al. 1993;

Vulpe et al. 1993; Petrukhin et al. 1994). MNK is found in the cell plasma membrane. Mutations in this protein cause systemic Cu deficiency (Vulpe et al. 1993). WND is predominantly expressed in the liver and its mutations result in systemic overload of Cu due to the lack of hepatic clearance (Petrukhin et al. 1994).

The simplest, and presumably most ancient, ion pumps are P_{IB}-type pumps. The substrates of P_{IB}-type ATPases are heavy metal ions such as Cu⁺, Ag⁺, Cd²⁺, Cu²⁺, Zn²⁺, Pb²⁺, Co²⁺, etc. Hydrophobicity analysis of the sequence of P_{IB}-ATPases shows that they contain 6 to 8 transmembrane fragments (Argüello 2003). The topology of CadA from *H.pylori* (Melchers et al. 1996) and CadA from *S. aureus* (Tsai et al. 2002) has been experimentally determined, to have eight TMs.

One of the unique features of these proteins is the presence of a putative metal binding sequence “CPx” (CPC, CPH, or SPC) in the transmembrane domain immediately upstream of the phosphorylation site (Solioz and Vulpe 1996; Argüello 2003). The CPx motif was first demonstrated to be essential for the translocation of the metal ion when these Cys were mutated in *C. elegans* Cu-ATPase (Yoshimizu et al. 1998). Similar results were observed when the mutations in the CPC from CopA of *A. fulgidus* produce a loss of ATPase activity (Mandal and Argüello 2003). This conclusion was also confirmed in *E. hirae* CopB (Bissig et al. 2001) and *E. coli* CopA (Fan and Rosen 2002).

Another noticeable characteristic of P_{IB}-type ATPases is the presence of 1-6 metal binding domains in their N terminus (N-MBD). These cytoplasmic N-MBDs include either the consensus CXXC or a His-rich sequence. The capacity of domains containing the CXXC sequences to bind metals and to interact with soluble metal chaperones has been

established (Lutsenko et al. 1997; Rosenzweig and O'Halloran 2000). However, it was demonstrated that these motifs are not essential for enzyme activity or for the metal ion selectivity process. Either removal of these domains or point mutations in the Cys of the CXXC consensus sequence did not suppress completely protein activity and N-MBDs are not required for ion transport; therefore, these domains were proposed to have regulatory roles (Voskoboinik et al. 1999; Mitra and Sharma 2001; Voskoboinik et al. 2001; Mana-Capelli et al. 2003; Mandal and Argüello 2003).

One striking feature about P_{1B} -ATPases is that, while sharing similar consensus sequences, their specificities towards metal ions differ greatly. Therefore, it is apparent that the specificity determinants lie somewhere else in the protein structure. Based on sequence homology and similarity to functionally characterized P_{1B} -ATPases, these enzymes were subdivided in six groups, IB1-6. Each subgroup presents a characteristic N terminal metal binding domain (N-MBD), a typical sequence in H6 plus signature sequences in H7 and H8, associated with their metal specificities (Argüello 2003). The structural characteristics of each subgroup are summarized in Table 1.

Subgroup	Metal specificity	N-MBD	H6	H7	H8
1B-1	Cu ⁺ /Ag ⁺	0-6 CXXC	CPC	NX ₆ YNX ₄ P	MX ₂ SSX ₅ [N/S]
1B-2	Zn ²⁺ /Cd ²⁺ /Pb ²⁺	0-2 CXXC + (HX) _n	CPC	NX ₇ K	DXGX ₇ N
1B-3	Cu ²⁺ /Cu ⁺ /Ag ⁺	H-rich	CPH	NX ₅ GYNX ₄ P	PXMSXSTX ₅ N
1B-4	Co ²⁺	-	SPC	not-identified	HEG[G/S]TX ₅ [N/S][G/A/S]
1B-5	?	-	TPC	not-identified	not-identified
1B-6	?	?	?	not-identified	not-identified

Table 1: Structural characteristics of the six subgroups in the P_{1B} ATPase subfamily.

1.5. Ion-Transport Mechanism

The P_{1B}-type ATPases function can be described by the catalytic cycle similar to all P-type ATPases, changing conformation between states E₁ and E₂ (Inesi 1985; Moller et al. 1996; Axelsen and Palmgren 1998). This cycle is characterized by the coupled reaction of ion translocation and ATP hydrolysis (Glynn 1985). The transport process consists of a sequence of steps: ion binding, ion occlusion, conformational transition of the protein, successive deocclusion of the ions, and ion release to the other side of the membrane. Cytoplasmic ions bind to a high-affinity site in the ATPase E₁ state. Ion binding triggers phosphorylation of the conserved Asp residue in the DKTGT sequence of the enzyme by Mg²⁺-ATP. The transition to the phosphorylated E₂-P state with a reduced affinity for ions, allows the escape of ions to the other side of the membrane. Finally the phosphate group is

hydrolyzed and the enzyme returns to its original state (Fig. 2) (Glynn 1985; Inesi 1985; Kuhlbrandt 2004).

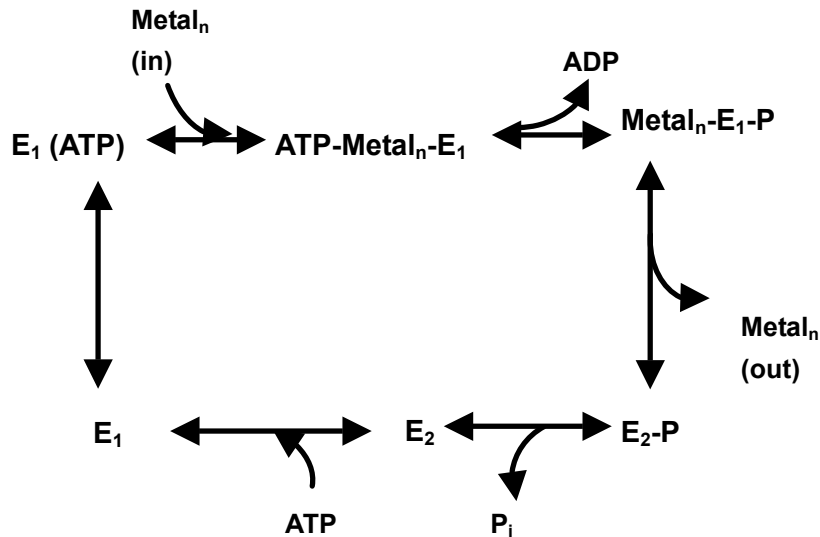


Fig. 2. Scheme of the catalytic cycle of P_{1B}-type ATPases. E₁ and E₂ are conformations of the ion pump with ion-binding sites facing the cytoplasm and extracellular medium, respectively. The metal ion is translocated using the energy of generated by ATP hydrolysis, which triggers conformational changes of the enzyme. Enzyme phosphorylation and dephosphorylation occur on the cytoplasmic side of the protein.

1.6. P_{1B}-ATPases in *Archaeoglobus fulgidus*

CopA gene comes from a thermophilic archaeae *Archeoglobus fulgidus*, for which its entire genome has been sequenced (Klenk et al. 1997). Thermophilic proteins and enzymes might serve as models to study due to their property of inherent stability that allows them to work at high temperatures (van de Vossenberg et al. 1998; Mandal et al. 2002).

Archaeoglobus fulgidus presents two P_{1B}-ATPases, CopA and CopB. CopB contains His residues at both metal binding sites; a His-rich motif (which includes seventeen His residues) as N-MBD and the “CPH” motif in the H6 transmembrane helix. Our group has demonstrated that this enzyme is a Cu²⁺-ATPase. The His-rich domain of CopB is not

involved in the selectivity of the metal ion (Mana-Capelli et al. 2003).

Gene *AF0473* encodes the CopA enzyme; a thermophilic ATPase which selectively drives the outward transport of Cu^+ or Ag^+ (Mandal et al. 2002) (Fig. 3). Our laboratory has described the heterologous expression and purification of CopA in *E. coli* as a fusion protein with a hexahistidine tag using a Ni^{2+} affinity column (Mandal et al. 2002). The enzyme has a maximal activity at 75° C, 400 mM NaCl, pH 6.1 and 20 mM Cys. The ATPase activity of CopA was stimulated by the monovalent cations Cu^+ ($V_{\max} = 3.66 \mu\text{mol/mg/h}$ and a $K_{1/2} = 2.1 \mu\text{M}$) and in a higher extent by Ag^+ ($V_{\max} = 14.82 \mu\text{mol/mg/h}$ and a $K_{1/2} = 29.4 \mu\text{M}$). Divalent Cu^{2+} or Zn^{2+} could not act as substrates. This demonstrates that this Cu-translocating P-type ATPase distinguishes between Cu^+ and Cu^{2+} . The enzyme also showed interaction with ATP with two apparent affinities (ATPase $K_{1/2} = 0.25 \text{ mM}$ and phosphorylation $K_m = 4.81 \mu\text{M}$). The phosphoenzyme levels in presence of both metal ions were measured using $[\gamma\text{-}^{32}\text{P}] \text{ATP}$, showing similar levels of phosphorylation. The presence of vanadate leads to enzyme inactivation ($\text{IC}_{50} = 24 \mu\text{M}$).

Our group described the functional roles of metal binding domains of CopA using site directed mutagenesis of Cys in the transmembrane CPC site and the CxxC consensus in N-MBD and C-MBD (Mandal and Argüello 2003). Replacement of transmembrane Cys removes enzyme activity and phosphoenzyme formation by ATP in the presence of metals, supporting their central role in ion transport. Mutations of Cys in the N-terminal cytoplasmic metal binding domains only slightly reduced the enzyme turnover suggesting a regulatory role for this domain. Cys mutation of C-terminal metal binding domain has no effect in enzyme activity.

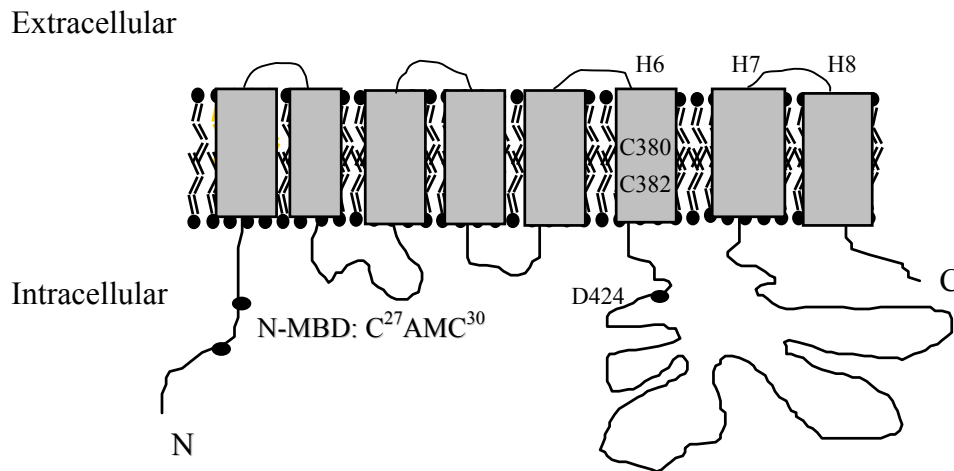


Fig. 3. Scheme of *A. fulgidus* CopA membrane topology. Asp424 is phosphorylated during the enzyme catalytic cycle, and Cys380 and Cys382 have been proposed to be part of the transmembrane Cu^+ binding site (Mandal and Argüello 2003).

1.7. Transmembrane Metal Binding Site of *Archaeoglobus fulgidus* CopA

Metal transport by P-type ATPases requires the presence of the transmembrane metal binding sites (Glynn 1985). In the “Metal_n-E₁-P” enzyme conformations, occluded cations are bound within the structure of the protein and cannot exchange with cations in the media. Assuming a common mechanism, Cu^+ -ATPases should also have a transmembrane metal binding site during the metal transport. In the family of P-type ATPases, the transmembrane cation binding sites of Na, K-ATPase and the SR Ca-ATPase were investigated to much greater detail than all others (Clarke et al. 1989; Argüello and Lingrel 1995; Argüello et al. 1996; Kuntzweiler et al. 1996; Vilsen and Andersen 1998). In the case of SR Ca-ATPase, it was shown by the crystal structure that the transmembrane cation binding sites of Ca-ATPases were found to be inside about 30-40% of the transmembrane thickness from

the cytoplasmic surface (Toyoshima et al. 2000; Toyoshima and Nomura 2002). In the E₁ conformation, the two dehydrated Ca²⁺ ions are perfectly coordinated by the side chains of conserved amino acid in H4, H5 and H6. These transmembrane domains would be equivalent to H6, H7 and H8 in the Cu⁺-ATPases (Argüello 2003). Thus, Ca-ATPases provide a model to understand transmembrane metal binding sites in heavy metal ATPases.

The metal binding sequence “CPx” (CPC, CPH, or SPC) in H6 of P_{IB}-type ATPases appear as part of a coordinating site required for heavy metal transport (Solioz and Vulpe 1996; Fan and Rosen 2002; Mandal and Argüello 2003). However, Zn²⁺-ATPases and Cu⁺-ATPases transport different metals even though they share the same CPC consensus in their H6 (Fan and Rosen 2002; Mandal et al. 2002; Eren and Arguello 2004). Therefore, other residues must also interact with the metal to confer enzyme selectivity. Based on this hypothesis, our laboratory searched for consensus sequences in transmembrane fragments H7 and H8 (Argüello 2003). The analysis shows NX₍₆₎YNX₍₄₎PX_(5,25)PX₍₆₎MXXSSXXVXX[NS] is conserved in more than 100 P_{IB}-type ATPases. In this study, part of the work aimed to test whether these conserved residues participate in metal coordination. We performed the site-directed mutation on these and non-conserved residues in the H7-H8 transmembrane segments and afterwards these mutated proteins were expressed in *E. coli* and purified using Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin. The proteins were analyzed by ATPase assay to test for activity in the presence of Cu⁺ compared to wild type CopA (WT-CopA).

1.8. Interaction of Cu⁺ with Cys

Since Cu ions can easily cycle between Cu^{2+} and Cu^+ , some Cu complexes could take part into non-enzymatic redox processes. Thiols and in particular glutathione (GSH) are biomolecules, which can be oxidized by Cu-catalyzed process (Sprietsma 1999). The interaction between Cu and GSH has received much attention since the ratio between reduced and oxidized GSH functions as intracellular redox buffer. It is also reported to modulate the activity of thiol dependent enzymes, which possess crucial Cys residues sensitive to redox changes. Moreover, GSH coordinates Cu^+ with high affinity and GSH- Cu^+ complex is able to transfer Cu into protein active sites (Kamidate et al. 1996; Rigo et al. 2004).

In biological fluids, Cys concentration is quite high and the free Cu concentration is very low (<0.2 pM) due to the contribution of efficient chelators (Snedeker and Greger 1983; O'Halloran and Culotta 2000). Cu^+ ions can strongly bind to the thiol group of Cys forming polymeric species with bridging thiolate sulfur and stability constant of the order of 10^{10} M^{-1} (Rigo et al. 2004). Mitra and co-workers first observed activation of ZntA for Zn^{2+} in the presence of Cys (Sharma et al. 2000). In our laboratory, previous studies also showed that the presence of millimolar concentration of Cys can dramatically increase CopA Cu^+ -ATPase and *Arabidopsis* HMA2 Zn^{2+} -ATPase activity (Mandal and Argüello 2003; Eren and Arguello 2004). Other thiol reagents such as GSH or DDT were unable to stimulate these enzymes in similar way (DDT only partially substituted Cys as it is necessary for maintaining Cu in a reduced form). Taking into account a pK_{SH} of 8.33 and the high association constant ($10^{10} \text{ M} [\text{Cu}^+\text{-Cys}]$), amounts of Cys slightly over the metal

concentration would render practically all of the metal in the metal thiolate form and satisfy both equilibria. We have determined the dependence of CopA Ag-ATPase activity on the presence of Cys. However maximum activity was observed at 20 mM Cys (Mandal et al. 2002). Thus, considering that the enzyme shows a $2.1 \pm 0.3 \mu\text{M Cu}^+$ $K_{1/2}$ in the presence of 20 mM Cys, it suggests that a more intricate phenomenon than the simple dithiolate- or monothiolate-metal formation is required for enzyme activation by millimolar amounts of Cys. In this work, using two constructs, WT-CopA and truncated CopA lacking N-MBD (T-CopA), we carried out a kinetic characterization of the reaction cycle in order to: 1. Reveal the role that Cys plays in the delivery of metal ions to CopA transmembrane metal binding sites; 2. Test if the absence of N-MBD has any effect on the Cys-protein interaction.

2. METHODS

2.1. Cloning and Protein Expression

CopA cDNA cloned into pCRT7/NT-TOPO/His vector (Invitrogen, Carlsbad, CA) was used in this study (Mandal and Argüello 2003). BL21StarTM (DE3)pLysS *E. coli* cells (Invitrogen, Carlsbad, CA) carrying vector pSJS1240 encoding for rare tRNAs (tRNA^{arg}AGA/AGG and tRNA^{ile}AUA) (Kim et al. 1988) were used for expressing WT-CopA protein after induction with 0.75 mM isopropyl β -D-thiogalactopyranoside (IPTG). This vector introduces an N-terminal hexahistidine tag suitable for Ni²⁺ affinity purification. A second construct containing the Truncated CopA (T-CopA) (lacking the N-terminus 36 amino acids) was subcloned into pBADTOPO/His vector (Invitrogen, Carlsbad, CA). This vector introduces a carboxyl terminal hexahistidine tag suitable for Ni²⁺ affinity purification and a V5 epitope for immuno-detection. The gene sequence was confirmed by automated DNA sequence analysis. *E. coli* Top10+CP cells (Invitrogen, Carlsbad, CA) carrying an extra plasmid encoding for rare tRNAs (tRNA^{arg}AGA/AGG and tRNA^{ile}AUA) were transformed with this construct, grown at 37°C in 2 x YT media supplemented with 150 μ g/ml ampicillin, 50 μ g/ml chloroanphenicol and expression induced with 0.002% L-arabinose. Cells were harvested 3 h post-induction, washed with 25 mM Tris, pH 7.0, 100 mM KCl and stored at -70°C.

2.2. Protein Purification

Protein purification was carried out as previously described (Mandal et al. 2002). All purification procedures were carried out at 0-4 °C and no special precautions were taken to prevent enzyme oxidation. Cells were suspended in buffer A (25 mM Tris, pH 7.0, 100 mM sucrose, 1 mM phenyl methyl sulfonyl fluoride (PMSF)) and disrupted by passing them through a French Press 3 times at 20,000 p.s.i.. After addition of 0.02 mg/ml Dnase I, 2 mM MgCl₂, the homogenate was incubated for 30 min at 4°C. Lysed cells were centrifuged at 8,000 x g for 30 min. The supernatant was then centrifuged at 163,000 x g for 1 h, the pellet washed with buffer A, and centrifuged at 229,000 x g for 1 h. Membranes were resuspended in buffer A (10-15 mg/ml) and stored at -70°C. For protein solubilization and purification, membranes (3 mg/ml in buffer B: 25 mM Tris, pH 8.0, 100 mM sucrose, 500 mM NaCl, 1 mM PMSF) were treated with dodecyl-β-D-maltoside (DDM) (Calbiochem, San Diego, CA), added drop wise to a final concentration of 0.75%. The membrane preparation was incubated with the detergent for 1 h at 4°C with mild agitation. The suspension was cleared by centrifugation at 229,000 x g for 1 h and Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin (Quiagen, Valencia, CA) pre-equilibrated with buffer B plus 0.05% DDM, 5 mM imidazole was added to the supernatant. After incubation for 1 h at 4°C, the resin was placed on a column and washed with buffer B, 0.05% DDM, 20 mM imidazole. The protein was eluted with buffer B, 0.05% DDM and 200 mM imidazole. Fractions were concentrated by filtration in 50,000 MW centricon (Millipore, Billerica, MA) and imidazole was removed using a Sephadex G-25 column. The protein

was eluted with 25 mM Tris, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 10% glycerol and stored in this buffer at -70°C. All protein determinations were performed in accordance with Bradford (Bradford 1976). Protein expression and purification was examined on 10% SDS-PAGE (Laemmli 1970). Expression of the proteins was observed by staining the gels with Coomassie Brilliant Blue and immunoblotting using Anti-His₆ Epitope Tag Antibody (Rabbit Polyclonal IgG) and Donkey Anti-Rabbit IgG Antibody (horseradish peroxidase conjugate) (ABR, Golden, CO).

2.3. *ATPase Activity Assays*

ATPase activity assays were carried out as previously described (Mandal et al. 2002). ATPase activity assay mixture contained 50 mM Tris, pH (75 °C) 6.1, 3 mM MgCl₂, 3 mM ATP, 0, 2, 10, 20 or 50 mM Cys, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 0.01 mg/ml purified enzyme, and 100 μM Cu₂SO₄, 2.5 mM DTT. In different experiments these reagents were independently varied as indicated in the corresponding figures. The buffer was prepared at room temperature and pH at 75°C was calculated using pKa/°C conversion factors (Tris: -0.031). ATPase activity was measured for 10 min at 75°C. Released inorganic phosphate (P_i) was determined in accordance to Lanzetta et al (Lanzetta et al. 1979). Background activity, measured in the absence of transition metals, was less than 5% in all determinations.

2.4. *Phosphorylation Assays*

Enzyme phosphorylation with ATP was carried out in a medium containing 50 mM Tris, pH (25 °C) 7.5, 1 mM MgCl₂, 25 μM [γ -³²P] ATP, 0, 5, 10 or 20 mM Cys, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 20 % DMSO, 0.05 mg/ml purified enzyme and 100 μM CuSO₄, 2.5 mM DTT (Mandal et al. 2002). The reaction, performed at 25°C, was initiated by addition of [γ -³²P] ATP and stopped at indicated times with 5 volumes of ice-cold 10% trichloroacetic acid, 1 mM P_i. The samples were filtered through nitrocellulose 0.45 μm filters (Millipore, Billerica, MA), washed five times with acid stopping solution, and radioactivity measured in a scintillation counter.

2.5. E₂P Dephosphorylation Assays

The time course of enzyme forward dephosphorylation (E₂P→E₂+P_i) was performed using samples fully phosphorylated as described above. ATP at a concentration of 1 mM was added to the medium after 30 s instead of the acid stopping solution and the samples were incubated for another 3, 6, or 9 s at 25 °C. The incubations were stopped with 10% trichloroacetic acid containing 1 mM P_i. Samples were filtered and radioactivity measured as described above.

2.6. E₁P Dephosphorylation Assays

The time course of enzyme backward dephosphorylation (E₁P+ADP→E₁+ATP) was

examined in samples fully phosphorylated as described above. Samples were incubated on ice for 1 min before the addition of 1 mM ADP to the medium. These reactions were incubated for additional 3, 6, or 9 s at 0 °C before being stopped by the addition of acid. Samples were filtered and radioactivity measured as described above.

2.7. Data Analysis

Curves of ATPase activity versus Cu^+ or ATP, were fit to $v = V_{\text{max}} L / (L + K_{1/2})$, where L is the concentration of the variable ligand. Data analysis was done using the KaleidaGraph software (Synergy, Reading, PA). Experimental values are the mean \pm S.E. of at least three independent experiments performed in duplicate. The reported standard errors for V_{max} , K_m and $K_{1/2}$ are asymptotic standard errors reported by the fitting program.

3. RESULTS

3.1. Cloning and Expression of WT-CopA and T-CopA

Archaeoglobus fulgidus CopA cDNA cloned into pCRT7/NT-TOPO/His vector was used in this study (Mandal and Argüello 2003). T-CopA was subcloned into pBADTOPO/His vector and expressed in *E. coli* TOP10 carrying an extra plasmid encoding for rare codon tRNAs (see methods). Fig. 4 shows the presence of WT-CopA and T-CopA in the membrane fraction isolated from the corresponding cells after induction with IPTG or L-arabinose (Fig. 4, lanes 2 and 4). To purify the protein, the membrane fraction was solubilized with 0.75% DDM under conditions in which maximal enzymatic activities were retained. The solubilized proteins were subsequently purified by Ni²⁺-NTA affinity column (Fig. 4, lanes 3 and 5). These purification procedures yielded CopA proteins of >90% purity, as judged by densitometry of Coomassie Brilliant Blue-stained SDS-PAGE gel (Fig. 4, lane 3 and 5). Typically 7-10 mg of purified proteins was obtained per liter of cell culture.

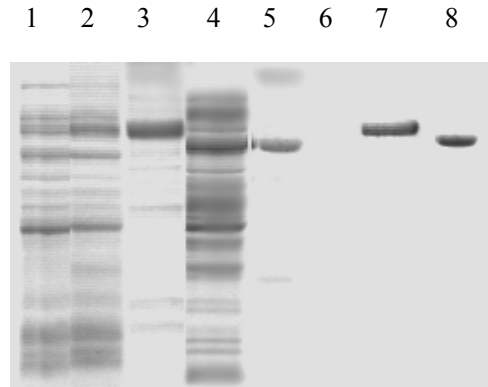


Fig. 4. Expression of WT-CopA and T-CopA mutant from *A. fulgidus*. Lanes 1 and 6: crude membrane fraction of uninduced *E. coli* transformed with pCRT7/NT-TOPO/His-CopA; lanes 2 and 4: IPTG or L-arabinose induced *E. coli* transformed with pCRT7/NT-TOPO/His-CopA and pBADTOPO-T-CopA respectively; lanes 3 and 7: purified WT-CopA; lanes 5 and 8: purified T-CopA. Lanes 1-5: 10% SDS-PAGE stained with coomassie brilliant blue; lanes 6-8: immunoblot with anti-His antibody.

3.2. Effect of Cys on WT-CopA and T-CopA Cu^+ -ATPase Activity

A. fulgidus CopA ATPase activity was shown to be strongly dependent on the Cys concentration with maximum enzymatic activities at Cys concentration of 20 mM (Mandal et al. 2002). In order to determine the role of Cys in Cu^+ -ATPase activity and to understand if the absence of the N-MBD had any effect on those parameters, Cu^+ -dependent ATPase activities were assayed for WT-CopA and T-CopA in the presence of various levels of Cys. Cu^+ ions bind strongly to the thiol group of Cys, forming polymeric species with bridging thiolate sulfur. The dissociation constant of these Cu^+ -Cys complexes are in the order of 10^{-10} M (Rigo et al. 2004). In the presence of millimolar levels of Cys, there will only be extremely low levels of free Cu^+ ($\sim 10^{-12}$ M) in the assay solution. The obtained results showed that Cys accelerates the Cu^+ dependent ATP hydrolysis of CopA (Fig. 5). Although

the Cu^+ $K_{1/2}$ for ATPase activation linearly correlates with the Cys concentration, Cys has a relatively small effect on Cu^+ affinity (Fig. 6). In addition, T-CopA exhibited similar V_{\max} and Cu^+ affinity to those of WT-CopA. This observation suggests that the N-MBD may not play a significant role during the Cu^+ delivery process.

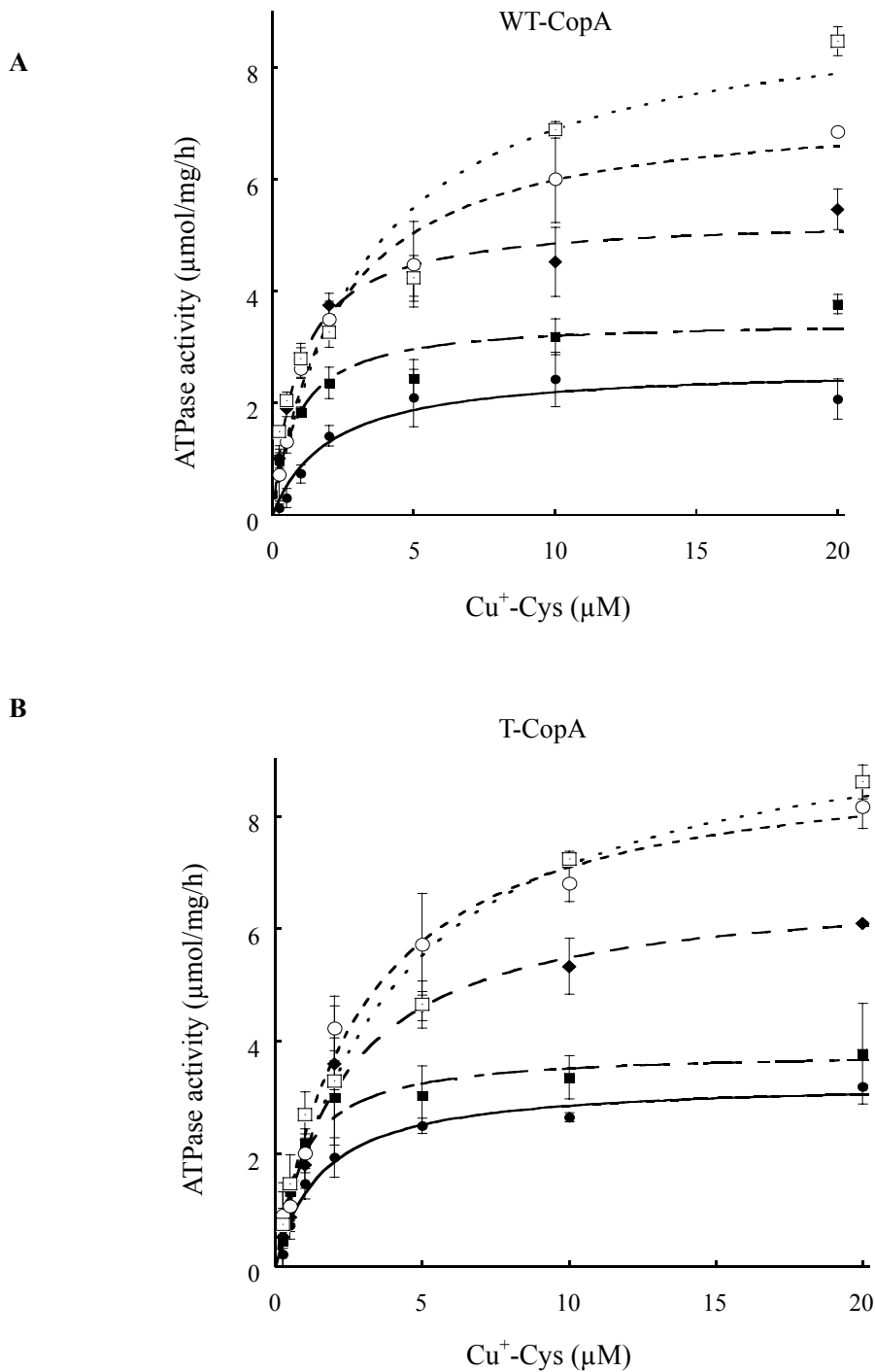


Fig. 5. Effect of Cys on WT-CopA and T-CopA Cu^+ -ATPase activity. The WT-CopA (A) and T-CopA (B) ATPase activities were measured in the presence of different concentrations of Cys (0 mM (●), 2 mM (■), 10 mM (◆), 20 mM (○), 50 mM (□)) and indicated concentrations of Cu^+ . Data were fitted to $v = V_{\text{max}} L / (L + K_{1/2})$ where L is the concentration of the $\text{Cu}^+\text{-Cys}$ complex. Points are the mean \pm SE of three independent experiments.

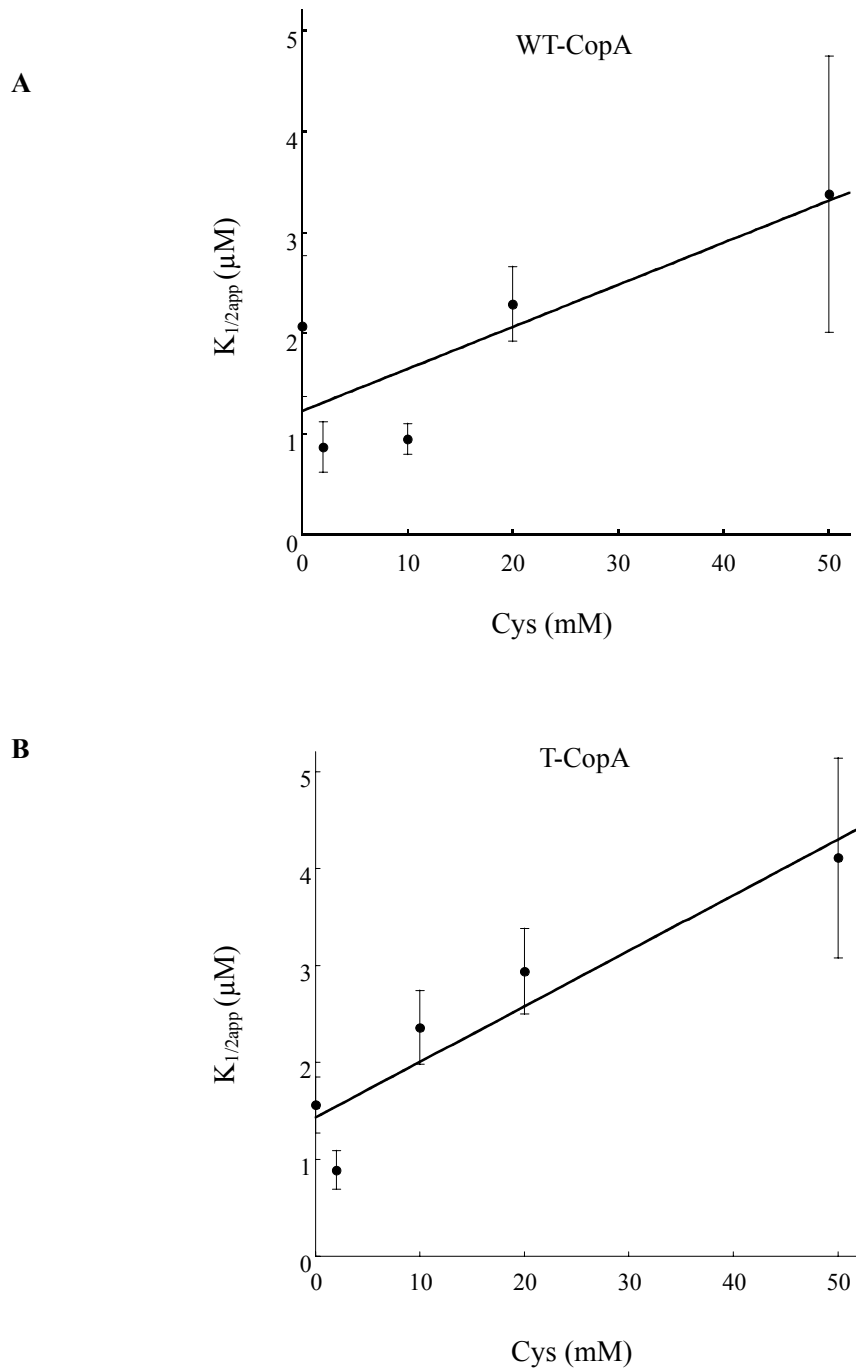


Fig. 6. Dependence of WT-CopA and T-CopA Cu^+ $K_{1/2app}$ on Cys. $K_{1/2app}$ versus [Cys] plots of WT-CopA (A) and T-CopA (B) derived from WT-CopA (Fig. 5A) and T-CopA ATPase activity curves (Fig. 5B) respectively. For WT-CopA, a linear fit to $K_{1/2app}$ yields a slope of 0.042 ± 0.017 and a y intercept of 1.23 ± 0.42 . For T-CopA, a linear fit to $K_{1/2app}$ yields a slope of 0.057 ± 0.012 and a y intercept of 1.43 ± 0.30 . Points are the mean \pm SE of three independent experiments.

3.3. Effect of Cys on WT-CopA and T-CopA Phosphorylation

In the catalytic cycle of P_{1B}-type ATPases, the high affinity interaction of P-type ATPases with ATP (μ M range) leads to phosphorylation of the enzyme at a conserved aspartic acid residue in the ATP binding site. We just showed that millimolar concentration of Cys activated the enzyme independently of its effect on Cu⁺ binding. To verify this hypothesis, we determined the phosphorylation rates and phosphorylation levels of CopA by adding difference concentrations of Cys. The temperature was lowered to 25° C to reduce turnover and 20% Me₂SO was added to the assay mix to stabilize the phosphorylated form of the enzyme. Our results showed that the steady state phosphorylation levels and the rates of WT-CopA positively correlated with the concentration of Cys (Fig. 7A, Fig. 8A). The fact that Cys stimulated P_{1E} steady-state level supported the idea of an activating effect of Cys independent of Cu⁺ binding. The absence of N-MBD didn't show significant effect on the phosphorylation rates/levels suggesting that enzyme activation by Cys is independent of the regulatory metal binding sites (Fig. 7B, Fig. 8B).

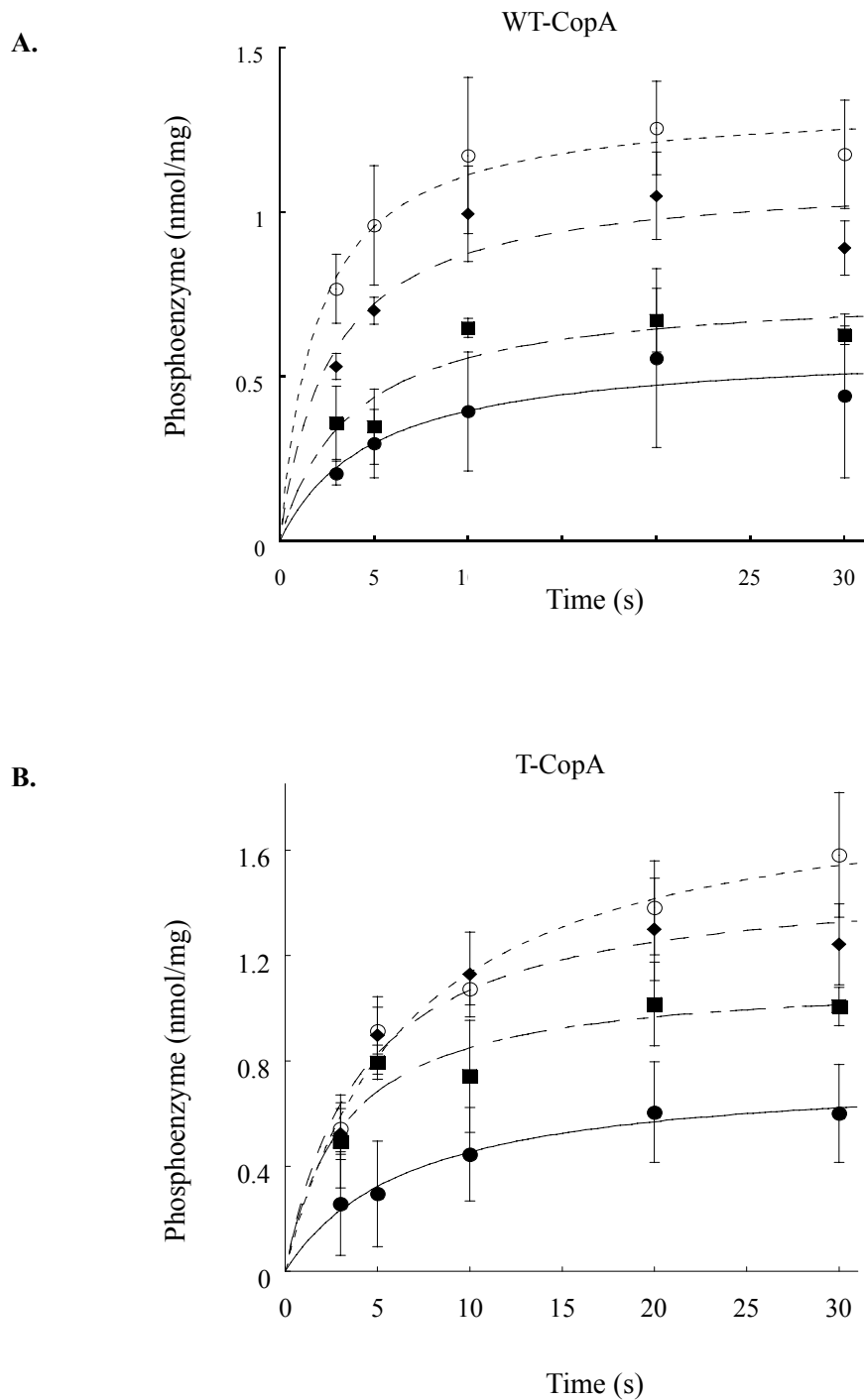
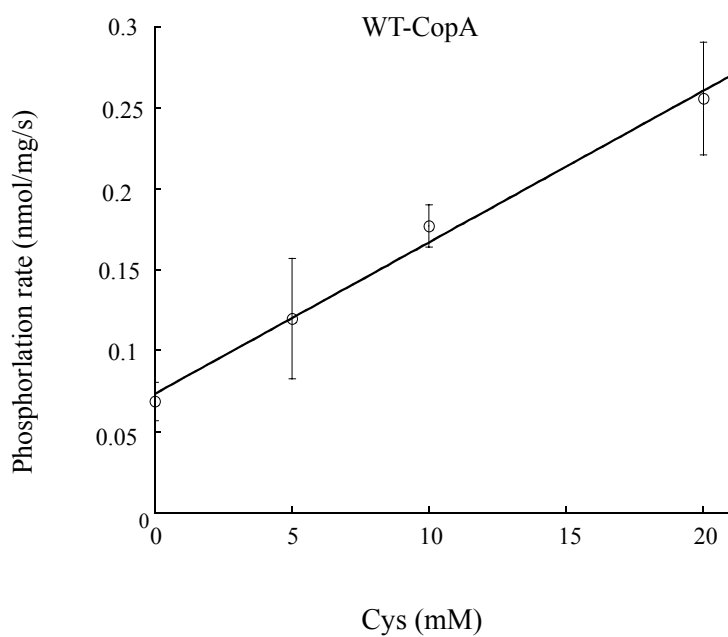


Fig. 7. Effect of Cys on WT-CopA and T-CopA phosphorylation. Phosphorylation of WT-CopA (A) and T-CopA (B) by $[\gamma\text{-}^{32}\text{P}]$ ATP in the presence of different concentrations of Cys (0 mM (●), 2 mM (■), 10 mM (◆), 20 mM (○), 50 mM (□)). The Cu^+ -activated enzyme phosphorylation by ATP was incubated at 25°C and the phosphorylation levels were measured at different time intervals. Data were fitted as described under “Experimental Procedures”. Points are the mean \pm SE of three independent experiments.

A.



B.

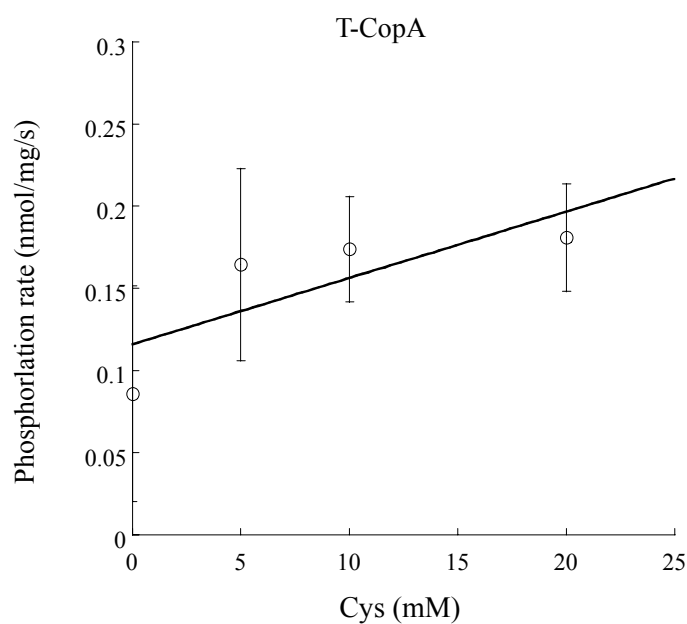
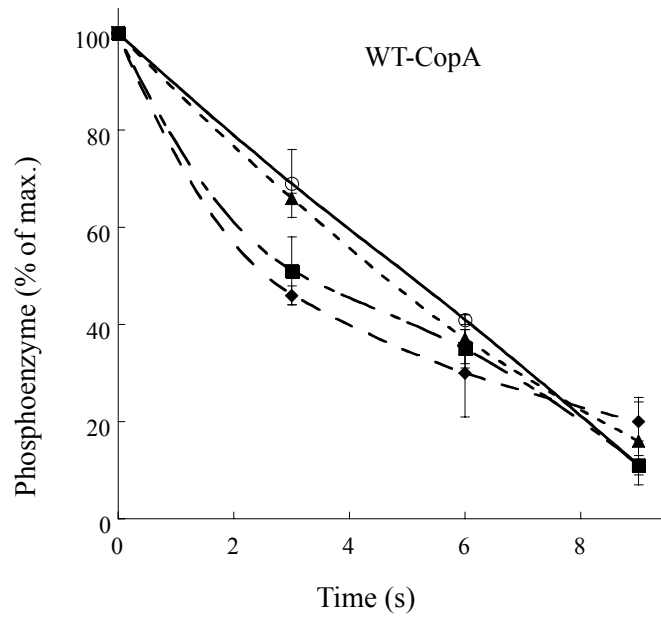


Fig. 8. Dependence of phosphorylation rate on Cys. Phosphorylation was calculated assuming a constant rate in the first 3seconds of phosphorylation. Plot A represents a linear fit of the WT-CopA initial velocity of phosphorylation and yields a slope of 0.009 ± 0.001 and a y intercept of 0.073 ± 0.007 . Plot B represents a linear fit of the T-CopA initial velocity of phosphorylation and yields a slope of 0.004 ± 0.002 and a y intercept of 0.116 ± 0.026 . Points are the mean \pm SE of three independent experiments.

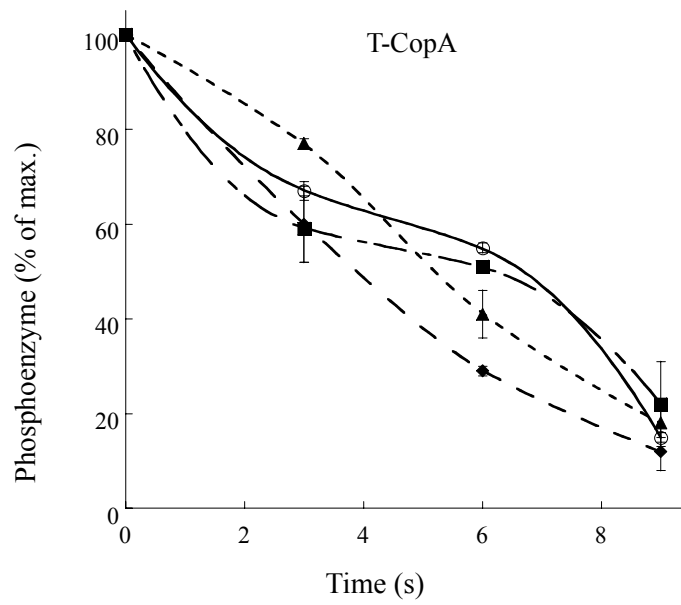
3.4. Effect of Cys on Forward (E_2P) and Backward (E_1P) Dephosphorylation of WT-CopA and T-CopA

We considered that Cys might influence the transition from E_2P to E_2 , shown to be the rate limiting step of the catalytic cycle (Mandal et al. 2002). In order to verify this hypothesis, we determined the forward dephosphorylation rate (dephosphorylation via E_2P after Cu^+ deocclusion) of CopA in the presence of different concentrations of Cys. Dephosphorylation experiments were carried out using Cu^+ activated WT-CopA or T-CopA phosphoenzyme. A concentration of ATP (1 mM) was used to urge the whole catalytic cycle forward and in turn push phosphoenzyme release P_i . Fig. 9A and Fig. 9B showed that Cys doesn't have apparent effect on forward dephosphorylation rates of WT-CopA or T-CopA. To test a possible effect of Cys on backward dephosphorylation rate (dephosphorylation via $E_1P \cdot Cu^+ + ADP \rightarrow E_1 + ATP + Cu^+$) of CopA this partial reaction was also characterized. To slow down the dephosphorylation rate for the convenience of detecting the possible effect of Cys, samples were incubated on ice for 1 min before the addition of 1 mM ADP to the medium. Cys doesn't show significant effect on the WT-CopA or T-CopA phosphoenzyme backward dephosphorylation rates (Fig. 9C and Fig. 9D).

A.



B.



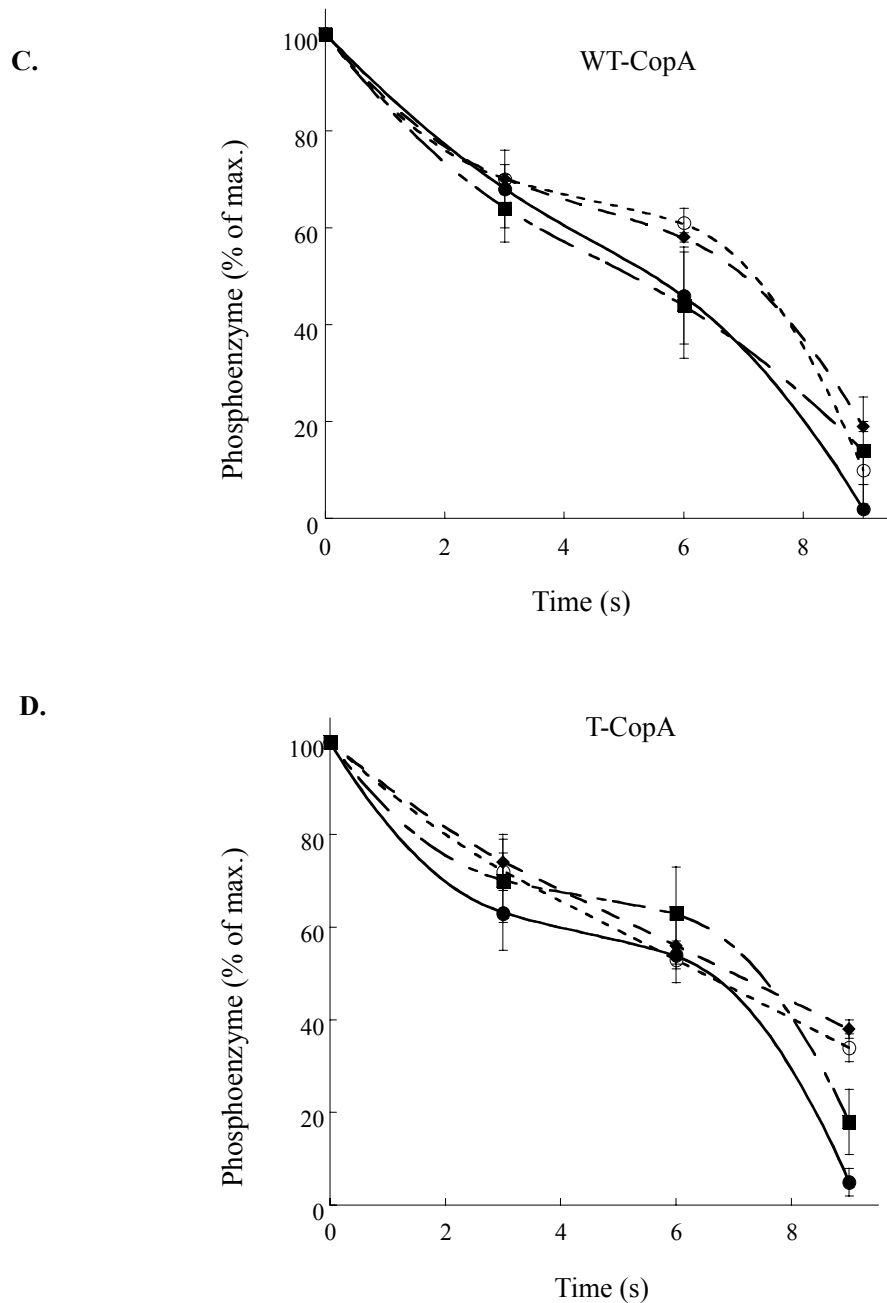


Fig. 9. Effect of Cys on forward (E_2P) and backward (E_1P) dephosphorylation of WT-CopA and T-CopA. WT-CopA (A, C) and T-CopA (B, D) were phosphorylated in the presence of $100 \mu\text{M Cu}^+$, $25 \mu\text{M } [\gamma\text{-}^{32}\text{P}] \text{ATP}$ and different concentrations of Cys (0 mM (\bullet), 2 mM (\blacksquare), 10 mM (\blacklozenge), and 20 mM (\circ)). 1 mM ATP was added to the medium after 30 s in enzyme forward dephosphorylation assays (A, B). 1 mM ADP was added to the medium after 30 s in enzyme backward dephosphorylation assays (C, D). No curve fitting was attempted. Points are the mean \pm SE of three independent experiments.

3.5. Effect of Cys on the Interaction of WT-CopA and T-CopA with ATP

In the catalytic cycle of CPx-ATPases, ATP interacts with two different apparent affinities (Mandal *et al.*, 2002). A low affinity interaction (sub-mM range) accelerates the conformational transition $E_2 \rightarrow E_1$ and as a consequence, the turnover. With the goal of obtaining the effect of Cys on this kinetic parameter and understand if the truncation of the protein has any affect, the dependence of ATPase on ATP was studied. Fig.10 shows WT-CopA exhibit similar apparent affinities towards ATP in the presence of Cys. This observation suggests that Cys has no significant effect on the $E_2 \leftrightarrow E_1$ equilibrium.

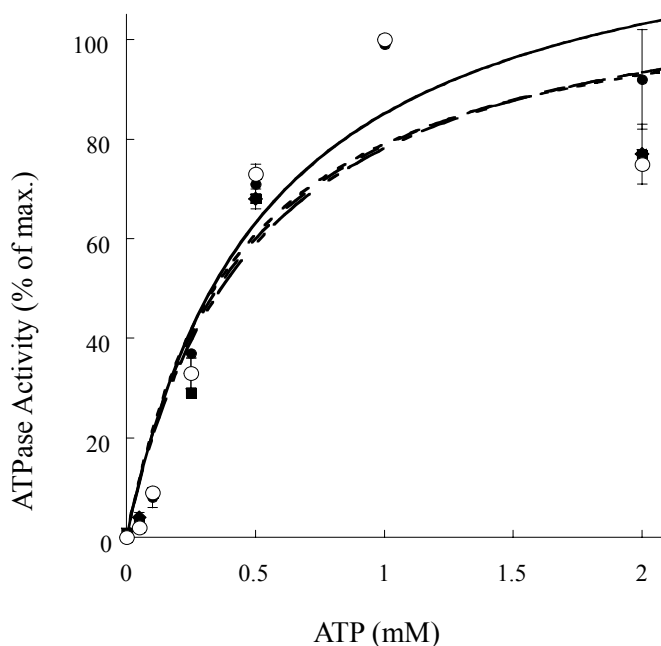


Fig. 10. Dependence of WT-CopA ATPase activity on ATP in the presence of Cys. The Cu^+ -ATPase activity was determined in the presence of different concentrations of Cys (0 mM (●), 2 mM (■), 10 mM (◆), and 20 mM (○)) and ATP concentration varied as indicated. The WT-CopA yielded an ATP $K_m = 0.4 \pm 0.02$ mM. Points are the mean \pm SE of two independent experiments.

3.6. Identification of Transmembrane Metal Coordination Residues Located in Cu⁺-transporting ATPases

Analysis of Cu⁺-ATPase sequences indicates that specific amino acids in their last two transmembrane segments are fully conserved and consequently might play important functional roles including metal coordination (Argüello 2003). In the *A. fulgidus* CopA sequence, these residues are Asn⁶⁷⁵, Tyr⁶⁸², Asn⁶⁸³, and Pro⁶⁸⁸ in H7 and Pro⁷⁰⁴, Met⁷¹¹, Ser⁷¹⁴, Ser⁷¹⁵ and Val⁷¹⁸ in H8. In addition, position 721 appears to require either Asn or Ser. They were subjected to conservative (Ser, Thr, Gln, or Cys) and nonconservative (Ala) replacements by site-directed mutagenesis, and the resulting proteins were functionally characterized. The ATPase activity results showed mutated proteins of Tyr⁶⁸², Asn⁶⁸³, Met⁷¹¹ and Ser⁷¹⁵, which are located in the central region of H7-H8 were largely inactive (Fig. 11). Considering their locations it can be proposed that these residues are probably responsible for Cu⁺ binding. Toward testing this hypothesis, it was relevant to determine the ability of the corresponding enzymes to interact with ligands and undergo catalytic conformational transitions. Partial reactions that are either Cu⁺-dependent (phosphorylation by ATP) or –independent (“backdoor” phosphorylation by P_i) supported this hypothesis. These mutations were not significantly phosphorylation by ATP or Cu⁺. However, they were able to undergo Cu⁺-independent phosphorylation, which means these mutations can form stable phosphor-intermediaries (Mandal et al. 2004). These observations further supported the postulated role of Tyr⁶⁸², Asn⁶⁸³, Met⁷¹¹ and Ser⁷¹⁵ probably participating in the Cu⁺ coordination during transport.

In addition, replacements of amino acids proximal to the cytoplasmic end of H7-H8, which are Asn⁶⁷⁵, Val⁷¹⁸, and Asn⁷²¹, also lack ATPase activity (Fig. 11). The observations of comparable phosphorylation level to WT in the presence of ATP and Cu⁺ but low phosphorylation by P_i suggested these mutants don't participate in metal coordination but their replacements probably "lock" the resulting enzymes in an E1 conformation, which prevents turnover at a measurable rate (Mandal et al. 2004). The homology model presented in Fig. 12 shows the spatial relation of putative Cu⁺ binding residues with other conserved amino acids in H7-H8. Homology modeling of CopA TMs was performed using the SR Ca-ATPase SERCA1a structure file as a template.

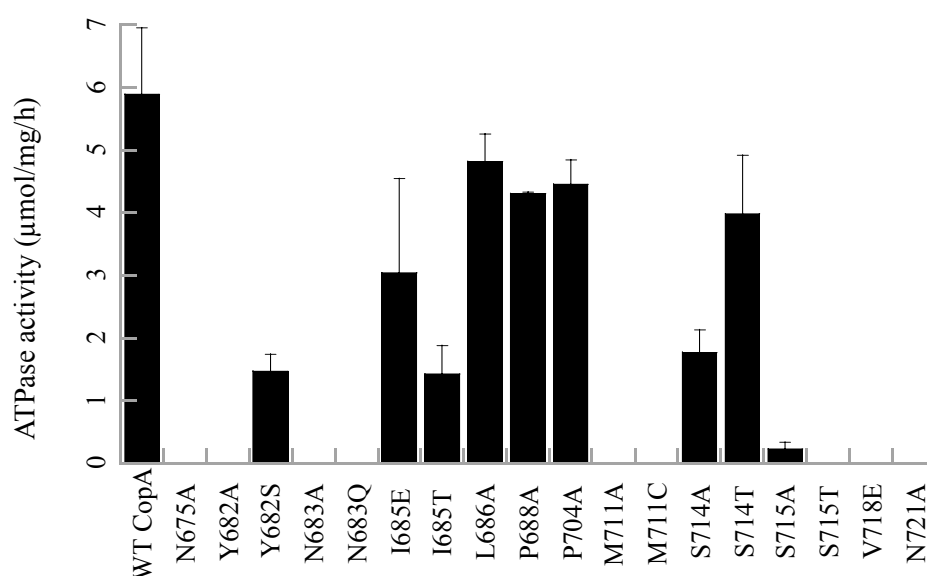


Fig. 11. Functional determination in amino acids mutated enzymes. The Cu⁺-ATPase activity was performed at 75° C in the presence of 20 mM Cys and 50μM Cu⁺. Data are the mean ± S.D. of 4 independent experiments.

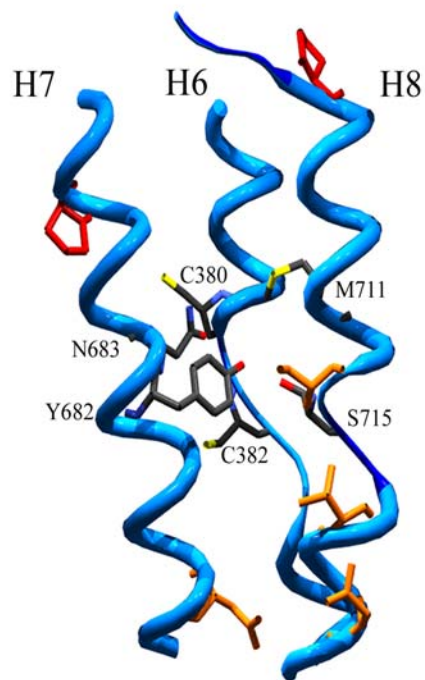


Fig. 12. Homology model of TMs H6-H8 of CopA based on the Ca-ATPase structure. Tyr⁶⁸², Asn⁶⁸³, Met⁷¹¹, and Ser⁷¹⁵ are shown using *CPK* color. Pro⁶⁸⁸ and Pro⁷⁰⁴ are shown in *red*. Asn⁶⁷⁵, Ser⁷¹⁴, Val⁷¹⁸, and Asn⁷²¹ are shown in *orange*.

4. DISCUSSION

In biological fluids, Cys concentration is quite high while the free copper concentration is very low (<0.2 pM) (Snedeker and Greger 1983; O'Halloran and Culotta 2000). Cys binds extremely tightly to Cu^+ ($\text{Cu-Cys } K_D < 10^{-10}$ M) (Rigo et al. 2004). The high affinity of CopA for Cu^+ ($2.0\text{-}3.5$ $\mu\text{M } \text{Cu}^+$) even in the presence of millimolar concentration of Cys suggests a multifaceted interaction of the enzyme with Cys. Cys was observed to dramatically increase ATPase activity in *E. coli* ZntA Zn^{2+} -ATPase, *A. fulgidus* CopA Cu^+ -ATPase and *Arabidopsis* HMA2- Zn^{2+} -ATPase (Sharma et al. 2000; Mandal et al. 2002; Eren and Arguello 2004). To elucidate the role Cys plays in metal transport, we studied the amino acid's effect on the partial reactions of the catalytic cycle of CopA. Our results suggest that while Cu^+ is delivered to the transport site as a Cu-Cys complex, Cys in the mM range stimulates the ATPase acting as a non-essential activator.

1. Cys functions as a non-essential activator of CopA

Taking into account Cys's $\text{p}K_{\text{SH}}$ of 8.33 and the Cu-Cys complex's very low dissociation constant ($K_D < 10^{-10}$ M), Cys at a slightly higher concentration than that of Cu^+ in the assay buffer should render all Cu^+ into the Cu-Cys complex form. Mitra and coworkers observed the highest V_{max} of ZntA at a metal ion:Cys ratio of 1:1 in their activity assays (Sharma et al. 2000; Mitra and Sharma 2001). These authors proposed that the metal-dithiolate or metal-monothiolate complexes might be the enzyme substrates. However, in our experiments the highest V_{max} values were obtained at a metal ion:Cys ratio of 1:1000. In the case of *Arabidopsis* HMA2- Zn^{2+} -ATPase, it also needed 50 mM Cys to obtain the

highest ATPase activity (Eren and Arguello 2004). These data suggest that while the formation of metal-dithiolate or metal-monothiolate complexes might as well be involved, a distinctive mechanism is in place for activating CopA and HMA2 by millimolar amounts of Cys. Excess amount of free Cys probably works as an activator that binds to allosteric sites of the transporter and in turn stimulates the phosphorylation of the enzyme.

In our experiment, Cys increased the ATPase activity of CopA but only moderately changed the apparent $K_{1/2}$ of Cu^+ from 1.5 μM to 3.0 μM . Cys seems to have no significant effect on enzyme's affinity to Cu^+ . Upon Cu-Cys complex approaching the transmembrane copper binding sites of the transporter, Cys could either dissociate with Cu^+ and pass the metal ion to the transporter, or remain bound with Cu^+ and get transported across the cell membrane as Cu^+ -Cys complex. Because all studies about P-type ATPases have shown to transport free ions across membrane assuming a common catalytic mechanism, we suspect that the second possibility is unlikely. Further experiments are necessary to solidly support this viewpoint.

2. Cys stimulates the ATPase activity of CopA by accelerating the phosphorylation of CopA

Considering that CopA still showed small amount of activity ($V_{\text{max}}=2\mu\text{mol}/\text{mg}/\text{h}$) in the absence of Cys, Cys in the mM range could be described as a non-essential activator of CopA.

Kinetic assays of Cu^+ -ATPase activity revealed that 2-50 mM Cys accelerates enzyme turn over with little effect on the Cu^+ affinity to CopA. The acceleration of ATP hydrolysis can be related to an increase of enzyme phosphorylation or forward dephosphorylation rates. Our follow-up experiments indeed demonstrated that Cys accelerated the

phosphorylation rate by four folds. Our results also showed that enzyme steady-state phosphorylation levels were elevated by four folds as well. Our experiments detected no enhancement of either the forward (E_2P) or the backward (E_1P) dephosphorylation rate of the enzyme in the presence of Cys. Based on these results, we can conclude that Cys stimulates the activity of CopA by accelerating the phosphorylation rates without affecting the dephosphorylation, therefore increasing steady-state levels of the enzyme.

Lipid vesicle based- ^{64}Cu uptake experiment showed that Cys acts on the cytoplasmic side of the enzyme, rather than the extracellular side (Mandal and Argüello unpublished results). This suggests that Cys facilitates Cu^+ ion's binding to, but not releasing from, the transmembrane metal binding site of CopA. This is consistent with our fact on that Cys didn't influence the forward dephosphorylation, which involves the release of Cu^+ to the extracellular media. Moreover, Cys doesn't affect $E_1 \leftrightarrow E_2$ equilibrium because it doesn't change ATP K_m . These observations have helped us narrowing the possible involvement of Cys from the entire catalytic cycle of CopA to the $E_1 \leftrightarrow E_1\text{-Cu}^+\text{-P}_i$ partial reactions.

Cys interact with the transporter independently of Cu^+ . It is unclear with which conformation of the enzyme Cys interacts and how such interaction influences the equilibrium between E_1 and E_2 conformations. Here, we propose an assay to determine the potential influence of Cys based on measuring the radioactive P_i incorporation into the enzyme via Cu^+ -independent "Backdoor" phosphorylation of CopA. Since E_2 has high affinity for P_i while E_1 has very low affinity for P_i . If Cys binds to E_1 , the phosphorylation level of the enzyme should be very low. However, our group observed relatively high "backdoor" phosphorylation level of WT-CopA in the presence of 20 mM Cys. We suspect

that Cys might preferentially bind to E₂ instead of E₁.

3. N-MBD is not involved in enzyme activation by Cys

In order to test whether the cytoplasmic MBD is involved in the role of Cys plays, CopA lacking the N-terminus domain (T-CopA) was assayed along with WT-CopA for enzyme activation, phosphorylation, and dephosphorylation. However, in all the assays we have performed, T-CopA showed undistinguishable kinetics from those of WT-CopA. We concluded that enzyme activation by Cys is independent of the regulatory metal binding sites in N-MBD.

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