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Activation of *Archaeoglobus fulgidus* Cu⁺-ATPase CopA by cysteine

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Abstract

CopA, a thermophilic ATPase from *Archaeoglobus fulgidus*, drives the outward movement of Cu⁺ across the cell membrane. Millimolar concentration of Cys dramatically increases (≈800%) the activity of CopA and other P_{1B}-type ATPases (*Escherichia coli* ZntA and *Arabidopsis thaliana* HMA2). The high affinity of CopA for metal (≈1 μM) together with the low Cu⁺-Cys K_D (<10⁻¹⁰M) suggested a multifaceted interaction of Cys with CopA, perhaps acting as a substitute for the Cu⁺ chaperone protein present *in vivo*. To explain the activation by the amino acid and further understand the mechanism of metal delivery to transport ATPases, Cys effects on the turnover and partial reactions of CopA were studied. 2–20 mM Cys accelerates enzyme turnover with little effect on CopA affinity for Cu⁺, suggesting a metal independent activation. Furthermore, Cys activates the p-nitrophenyl phosphatase activity of CopA, even though this activity is metal independent. Cys accelerates enzyme phosphorylation and the forward dephosphorylation rates yielding higher steady state phosphoenzyme levels. The faster dephosphorylation would explain the higher enzyme turnover in the presence of Cys. The amino acid has no significant effect on low affinity ATP K_m suggesting no changes in the E₁ ↔ E₂ equilibrium. Characterization of Cu⁺ transport into sealed vesicles indicates that Cys acts on the cytoplasmic side of the enzyme. However, the Cys activation of truncated CopA lacking the N-terminal metal binding domain (N-MBD) indicates that activation by Cys is independent of the regulatory N-MBD. These results suggest that Cys is a non-essential activator of CopA, interacting with the cytoplasmic side of the enzyme while this is in an E₁ form. Interestingly, these effects also point out that Cu⁺ can reach the cytoplasmic opening of the access path into the transmembrane transport sites either as a free metal or a Cu⁺-Cys complex.

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1. Introduction

Archaeoglobus fulgidus CopA is a thermophilic P_{1B}-type ATPase that selectively drives the outward transport of Cu⁺ or Ag⁺ [1,2]. P_{1B}-type ATPases, a subfamily of P-type ATPases, transport heavy metals (Cu⁺, Ag⁺, Cu²⁺, Zn²⁺, Cd²⁺, Pb²⁺, Co²⁺) across membranes using the energy provided by ATP [3,4]. They confer metal tolerance to microorganisms [5,6] and are essential for the absorption, distribution, and bioaccumulation of metal micronutrients by higher organisms [6–8]. Mutations in the two human Cu⁺-ATPases are responsible for the Menkes and Wilson's diseases [9].

Most P_{1B}-type ATPases, including *A. fulgidus* CopA, have eight transmembrane segments and a large cytoplasmic loop responsible for ATP binding and hydrolysis located between transmembrane fragments sixth (H6) and seventh (H7) [3,4,10–12]. Invariant residues in H6, H7 and transmembrane fragment eight (H8) constitute signature sequences that allow predicting the metal specificity of these enzymes [3]. In Cu⁺-ATPases, two Cys in H6, Tyr and Asn in H7 and Met and Ser in H8, are required for transmembrane Cu⁺ binding associated with metal translocation [2]. In addition, Cu⁺-ATPases have one to six cytoplasmic N-terminus metal-binding domains (N-MBDs) characterized by the sequence CXXC. These have high homology to soluble Cu⁺ chaperones, fold independently, and their structure has been established [13–15]. N-MBDs are not required for ion transport, but play a regulatory role by

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controlling enzyme turnover and, in eukaryotes, the targeting of Cu^+ -ATPases to various membrane compartments [14,16–18].

In vitro experiments have shown that Cu^+ -ATPases are activated by Cu^+ ions in the absence of chaperones or chelating molecules with $K_{1/2}$ in the low micromolar range [1,18–20]. However, it has been postulated that in vivo the free Cu concentration is probably in the nanomolar–picomolar range since most cytoplasmic Cu^+ is associated to specific metal chaperones [21]. The transfer of Cu^+ from specific Cu^+ -chaperones to Cu^+ -ATPase N-MBDs has been shown [14,17,22–25]. On the other hand, the Cu^+ -dependent phosphorylation of Wilson disease protein in the presence of Cu^+ -Atox1 and the absence of free Cu^+ supports the hypothesis that chaperones “deliver” the metal to the enzyme for transmembrane transport [24,26]. Yet, the metal delivery to transport sites is not fully understood.

Mitra and co-workers first observed activation of *Escherichia coli* ZntA, a Zn^{2+} -ATPase, by Cys and glutathione [27]. In our laboratory, we observed that the presence of millimolar concentration of Cys increases the turnover of *A. fulgidus* CopA and *Arabidopsis thaliana* HMA2, a Zn^{2+} -ATPase [1,28]. However, other antioxidants such as ascorbate or thiol reagents like glutathione or dithiothreitol were unable to stimulate CopA or HMA2. Cys appears to have no effect on *A. fulgidus* CopB, a Cu^{2+} -ATPase [29]. Since Cu^+ -ATPases can likely accept the free metal or the chaperone-delivered metal as substrate, it is tempting to hypothesize that in some enzymes Cu^+ -Cys might act as substrate replacing the Cu^+ -chaperone. However, additional information should be taken into consideration. Maximum Cu^+ -ATPase activity is observed in the presence of 20 mM Cys with Cu^+ $K_{1/2}$ = 2.3 μM for enzyme activation [1]. Taking into account a $\text{p}K_{\text{SH}}$ of 8.33 for Cys and the strong binding of Cu^+ to the Cys thiol groups (K_{D} 10^{-10} M) [30] and assuming relatively small changes in these constants at the assay temperature (75 °C), amounts of Cys slightly over the metal concentration would render practically all of the metal in the Cu^+ -thiolate form. Thus, it can be postulated that, while thiolate–metal forms might act as substrates of the Cu^+ -ATPase, Cys has additional stimulating effects when present in millimolar concentrations.

To test these hypotheses, we characterized the effects of Cys on the kinetics of *A. fulgidus* CopA Cu^+ transport, Cu^+ -ATPase and phosphatase activities. We observed that Cys, independently of the presence of metals, is a non-essential activator of CopA. Moreover, our experiments indicate that, although Cu^+ is likely transported as a free ion, the metal-thiolate can deliver the metal to the protein.

2. Materials and methods

2.1. cDNA constructs and protein expression

A. fulgidus CopA cDNA cloned into pCRT7/NT-TOPO vector (Invitrogen, Carlsbad, CA) was used in this study [16]. BL21Star™ (DE3) pLysS *E. coli* cells (Invitrogen, Carlsbad, CA) carrying the streptomycin resistant plasmid pSJS1240 encoding for rare tRNAs (tRNA^{arg}AGA/AGG and tRNA^{ile}AUA) [31] were used for expressing CopA protein after induction with 0.75 mM isopropyl β -D-thiogalactopyranoside. A second construct encoding a truncated

CopA lacking the first 70 amino acids, T-CopA, was subcloned into pBADTOPO/His vector (Invitrogen, Carlsbad, CA). This vector introduces a carboxyl terminal hexahistidine tag suitable for Ni^{2+} affinity purification. *E. coli* Top10 cells (Invitrogen, Carlsbad, CA) carrying the chloramphenicol resistant plasmid CP encoding for rare tRNAs (tRNA^{arg}AGA/AGG and tRNA^{ile}AUA) [31], were transformed with this construct and expression induced with 0.002% L-arabinose.

2.2. Enzyme preparation

Enzyme was prepared as described [1]. Briefly, membranes were isolated from cells expressing the CopA or T-CopA 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. After purification, proteins were stored in 25 mM Tris, (pH 8.0), 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 1 mM dithiothreitol (DTT) at –80 °C. All protein determination were performed in accordance to Bradford [32].

2.3. ATPase activity assays

ATPase activity determinations were carried out in a medium containing: 50 mM Tris–HCl (pH 6.1 at 75 °C), 3 mM MgCl_2 , 3 mM ATP, 20 mM Cys, 0.01% aroclorin, 0.01% DDM, 400 mM NaCl, 100 μM CuSO_4 , 2.5 mM DTT and 0.01 mg/ml purified enzyme [1]. In various experiments, some of these reagents were independently varied as indicated in the corresponding figures. ATPase activity was measured for 10 min at 75 °C. Released inorganic phosphate (P_i) was determined in accordance to Lanzetta et al. [33].

2.4. p-nitrophenyl phosphatase (pNPPase) activity assays

pNPPase activity was determined in a medium containing: 60 mM p-nitrophenyl phosphate (pNPP), 50 mM MOPS-K (pH 6.1 at 75 °C), 3 mM MgSO_4 , 0.01% aroclorin, 0.01% DDM, 0.014 mg/ml purified CopA, and various Cys concentrations. pNPPase activity was measured for 10 min at 75 °C. pNPP hydrolysis was estimated by measuring the absorption of p-nitrophenol at 410 nm after adding 4 volumes of 0.5 N NaOH (ϵ = 17000 $\text{M}^{-1} \text{cm}^{-1}$).

2.5. Phosphorylation assays

Enzyme phosphorylation by ATP-Mg was carried out as previously described [1] in a medium containing: 50 mM Tris (pH 7.5 at 25 °C), 1 mM MgCl_2 , 25 μM [γ - ^{32}P] ATP, 0.01% aroclorin, 0.01% DDM, 400 mM NaCl, 20% DMSO, 100 μM CuSO_4 , 2.5 mM DTT, 0.05 mg/ml purified enzyme, and Cys as indicated in the corresponding figures. The reaction, performed at 25 °C, was initiated by addition of [γ - ^{32}P] ATP and stopped at indicated times with 5 volumes of ice-cold 10% trichloroacetic acid, 1 mM P_i (acid stopping solution). The samples were filtered through 0.45 μm nitrocellulose filters, washed five times with acid stopping solution, and radioactivity measured in a scintillation counter.

2.6. Dephosphorylation assays

The time course of E_2P enzyme dephosphorylation ($\text{E}_2\text{P} \rightarrow \text{E}_2 + \text{P}_i$) was monitored using samples phosphorylated as described above. After 30 s incubation, dephosphorylation was initiated by adding 1 mM ATP to the medium. Samples incubated for 3, 6, or 9 s at 25 °C and the reaction terminated with acid stopping solution. The time course of E_1P enzyme dephosphorylation ($\text{E}_1\text{P} + \text{ADP} \rightarrow \text{E}_1 + \text{ATP}$) was examined in samples phosphorylated as described above. Samples were incubated at 0–4 °C for 1 min before starting dephosphorylation by adding 1 mM ADP to the medium. The dephosphorylation reaction was continued for 3, 6, or 9 s at 0 °C and ended by adding acid stopping solution. Samples were filtered and radioactivity measured as described above.

2.7. Sealed vesicles preparation

Sealed vesicles were prepared in accordance with Tsai et al. [34]. Cells were resuspended in 50 mM MOPS-K (pH 7.0), 250 mM sucrose, 200 mM KCl and

10 mM MgSO₄ at a concentration of 0.2 g cell/ml. 1 mg/ml Lysozyme was added and the cell suspension was incubated at 37 °C for 1 h with gentle shaking. Before lysis, protoplasts media was made either 20 mM Cys or 0.1 mM bathocuproine disulphonate (BCS), 2.5 mM DTT. Protoplasts were lysed by passing through French Press at 10,000 psi. Cells debris were pelleted by centrifugations at 12,000×g for 1 h, and the supernatant centrifuged at 160,000×g for 1 h. Vesicles were resuspended in 50 mM MOPS-K (pH 7.0), 250 mM sucrose, 200 mM KCl and 10 mM MgSO₄, and stored at 4 °C.

2.8. Cu⁺ transport assays

Cu⁺ transport into the sealed vesicles was measured using ⁶⁴Cu (Mallinckrodt Institute of Radiology, Washington University Medical School). Membrane vesicles (1 mg protein/ml) were assayed in 50 mM Bis-Tris (pH 6.0 at 55 °C), 3 mM MgCl₂, 400 mM NaCl, 20 mM Cys, 2.5 mM DTT and 10 μM [⁶⁴Cu]CuSO₄ at 55 °C as described by Mana-Capelli et al. [29]. At this temperature, the enzyme has a significant, easily measurable, activity while minimum disruption of the vesicles integrity is observed. pH at 55 °C was calculated using a pKa/°C conversion factor of −0.0116 for Bis-Tris. Tubes were pre-incubated at 55 °C for 1 min and the reactions were started upon addition of 3 mM ATP. Aliquots were taken at the specified times and filtered onto 0.2 μm nitrocellulose filters. Filters were washed with ice-cold 25 mM Tris–HCl, (pH 7.0), 0.2 M KCl, 0.25 M sucrose, 0.1 mM CuSO₄ and radioactivity was measured in a scintillation counter.

2.9. Data analysis

Curves of ATPase activity versus Cu⁺, Cys, or ATP, were fit to $v = ((V_{max} - v_o) L / (L + K_{1/2})) + v_o$, 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 ± SE of 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

Our initial study showed that *A. fulgidus* CopA is activated by Cys [1]. CopA is activated by Ag⁺ or Cu⁺ ions and both, the Cu⁺- and Ag⁺-ATPase activities, are activated approximately 800% by Cys with a $K_{1/2}$ of 4 ± 1 mM (see Fig. 1 in Ref. 1). This activation was not observed when N-acetylcysteine was included in the assay media (not shown). A starting hypothesis to explain CopA activation by Cys is that a Cys–metal complex might act as a substrate of the enzyme. If this is the case, differences in the metal dependence of CopA enzymatic activity (i.e., Cu⁺ $K_{1/2}$ for activation) might be observed in the presence and absence of Cys. Fig. 1A shows the Cu⁺ dependence of CopA in absence and the presence of various Cys concentrations. It should be noticed here that the plotted Cu⁺ concentration is the total concentration in the system, not the levels of the free ion. While the enzyme activation by Cys was evident at all metal concentrations, no statistically significant change in Cu⁺ dependence was observed. This is shown in Fig. 1B, where the Cu⁺ $K_{1/2}$ for ATPase activation is plotted versus the Cys concentration in the assay media. These data would suggest that Cys does not activate the enzyme by affecting the access of metal to the transmembrane metal binding site. This transmembrane binding site is the one that must be occupied to drive the coupled ATP hydrolysis.

An alternative hypothesis to explain the activation by Cys might consider the vectorial interaction of P-type ATPases with the transported substrates. Then, it might be postulated that Cys

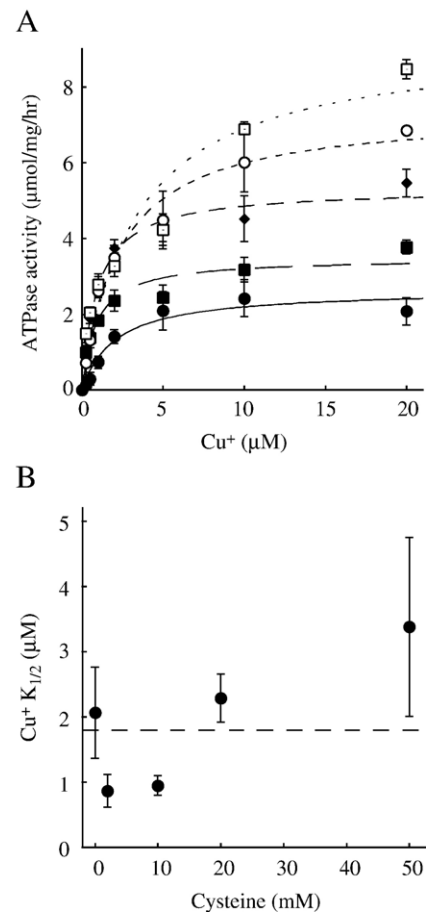


Fig. 1. Effect of Cys on CopA Cu⁺-ATPase activity. (A) ATPase activity was measured in the presence of various Cys concentrations and indicated levels of Cu⁺. Curves were fitted resulting in V_{max} (μmol/mg/h), $K_{1/2}$ (μM): 2.6 ± 0.2, 2.1 ± 0.6, 0 mM Cys (●); 3.5 ± 0.2, 0.9 ± 0.2, 2 mM Cys (■); 5.3 ± 0.2, 0.9 ± 0.1, 10 mM Cys (◆); 7.3 ± 0.4, 2.3 ± 0.4, 20 mM Cys (○); and 9.2 ± 1.2, 3.4 ± 1.3, 50 mM Cys (□). (B) Resulting $K_{1/2}$ for Cu⁺ activation of CopA ATPase activity at the indicated Cys concentrations. Points are the mean ± SE of three independent experiments.

could affect the metal release from the extracellular-facing metal binding sites in the transmembrane region. Testing this, the effects of Cys acting at the cytoplasmic and extracellular sides of the enzyme were explored by measuring Cu⁺ transport into sealed inside-out vesicles loaded with 0 mM Cys, 20 mM Cys, or 0.1 mM BCS, 2.5 mM DTT. In these experiments a mix population of right-side-out and inside-out vesicles was used. In these vesicles, only enzymes with the ATP binding–cytoplasmic side facing out participate in metal transport. Fig. 2A shows that the presence of Cys or other metal chelating reagents such as BCS inside the vesicles (extracellular side of transporting enzymes), has no significant effect on the rate of Cu⁺ transport by CopA. However, the presence of Cys in the cytoplasmic side has a significant stimulating effect (600%–800%) on the transport kinetics (Fig. 2B). This, again, was independent of metal chelators present inside the vesicles.

Many P_{1B}-type ATPases contain cytoplasmic metal binding domains. Although the regulatory role of N-MBDs is supported

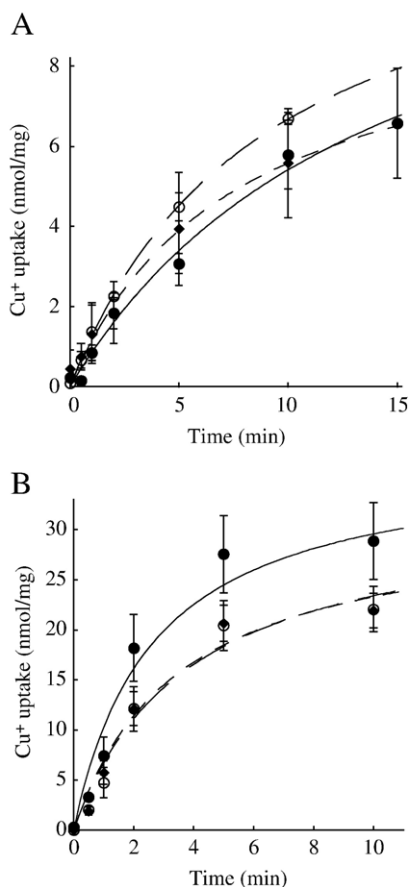


Fig. 2. Effect of Cys on Cu^+ uptake into sealed vesicles from *E. coli* expressing *A. fulgidus* CopA. Cu^+ uptake was determined in the absence (A) or in the presence (B) of 20 mM Cys outside of sealed vesicles prepared under various conditions yielding vesicles: not loaded with Cys (●), loaded with 20 mM Cys (○), or 0.1 mM BCS, 2.5 mM DTT (◆). Points are the mean \pm SE of three independent experiments.

by experimental evidence [14,16–18], an initial interaction of metals with N-MBDs and subsequent transfer to transmembrane sites has been suggested as a mechanistic alternative for metal transport. Considering this, the possible participation of CopA N-MBD in the enzyme activation by Cys was tested using a truncated CopA form lacking the N-MBD, T-CopA. This enzyme was also activated by Cys with a $K_{1/2}$ (10 ± 4 mM) similar to that observed for the wild type protein (compare Fig. 3 with Fig. 1A). Likewise, the Cu^+ $K_{1/2}$ for activation of the truncated enzyme was independent of the presence of Cys (not shown). These results indicate that CopA activation is not through an interaction of Cys (or Cys– Cu^+) with the N-MBD.

Moreover, the effects of Cys on T-CopA activity further suggest that Cys effects might be independent of metals–thiolate speciation. To test this possibility, analysis of the effects of Cys on a CopA metal-independent enzymatic activity was required. The capability of P_{II} -type ATPases (Na,K-ATPase, Ca-ATPase, etc.) to hydrolyze phenyl and acylphosphates (pNPP, acetyl phosphate, etc.) in a cation independent manner has long been established [35–37]. P_{IB} -type ATPases also show pNPPase activity (Fig. 4). As in the case of P_{II} -type ATPases, this activity is not stimulated by the phosphorylation driving

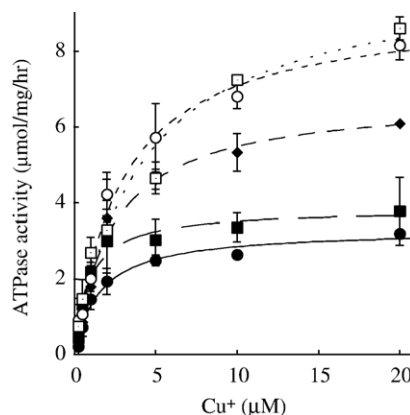


Fig. 3. Effect of Cys on T-CopA Cu^+ -ATPase activity. ATPase activity was measured in the presence of various Cys concentrations and indicated levels of Cu^+ . Curves were fitted resulting in V_{max} ($\mu\text{mol/mg/h}$), $K_{1/2}$ (μM): 3.3 ± 0.2 , 1.6 ± 0.3 , 0 mM Cys (●); 3.8 ± 0.2 , 0.8 ± 0.2 , 2 mM Cys (■); 6.8 ± 0.3 , 2.5 ± 0.3 , 10 mM Cys (◆); 9.2 ± 0.4 , 2.9 ± 0.4 , 20 mM Cys (○); and 10.0 ± 0.9 , 4.1 ± 1.0 , 50 mM Cys (□). Points are the mean \pm SE of three independent experiments.

metal, Cu^+ in the case of CopA (not shown). Relevant to the focus of this report, this pNPPase activity was activated ($\approx 800\%$) by Cys with a $K_{1/2} = 2.9 \pm 0.5$ mM. These parameters are quite similar to that observed for the stimulation of the Cu^+ -ATPase and, consequently, they suggest that the Cys effects are independent of the presence of metal substrates.

As previously shown, P_{IB} -type ATPases follow the well-described Albert–Post catalytic cycle [1,2,6,16,20,27,29,36–38]. Within this, the metal release and subsequent dephosphorylation appear as the rate limiting steps [16]. Thus, it could be hypothesized that Cys should accelerate these reactions. Testing this, the effects of Cys on CopA phosphorylation and dephosphorylation reactions were explored. Fig. 5A shows the kinetics of Cu^+ -dependent phosphorylation of CopA by ATP-Mg under conditions that facilitate the isolation of the phosphoenzyme intermediary (low ATP, 25 °C and 20% DMSO) in the presence of various Cys concentrations. This shows that increasing concentrations of Cys led to higher steady

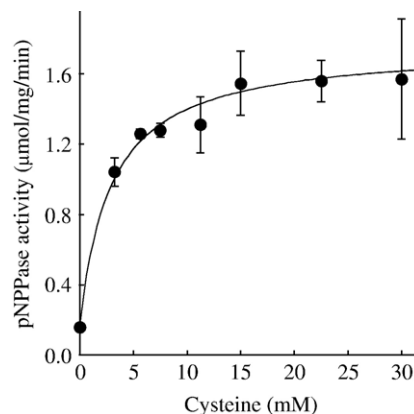


Fig. 4. Effect of Cys on CopA pNPPase activity. pNPPase activity was measured in the presence of various Cys concentrations. Curves were fitted resulting in $V_{\text{max}} = 1.77 \pm 0.05$ $\mu\text{mol/mg/min}$, $v_0 = 0.16 \pm 0.07$ $\mu\text{mol/mg/min}$, and Cys $K_{1/2} = 2.9 \pm 0.5$ mM. Points are the mean \pm SE of three independent experiments.

state phosphoenzyme levels and it would suggest that Cys interacts with the E1 form of the enzyme accelerating its phosphorylation. However, the phosphoenzyme level is the resultant not only of the rate of enzyme phosphorylation ($E1 + ATP + Cu^+ \rightarrow E1P(Cu^+)$), but also the rates of forward dephosphorylation ($E1P(Cu^+) \leftrightarrow E2P_i + Cu^+ \rightarrow E2 + P_i$) and reverse dephosphorylation ($E1P(Cu^+) + ADP \rightarrow E1 + ATP + Cu^+$). To estimate these parameters, E1P and E2P dephosphorylation were monitored in function of time and linearity was assumed in the initial reactions rates up to 3 s (time of earliest measurement). Fig. 5B shows the relationship between the estimated phosphorylation and dephosphorylation rates and Cys concentration. The slopes of the curves indicate that millimolar Cys accelerates CopA phosphorylation and does not affect the rate of the reverse E1P dephosphorylation. This would explain the larger phosphoenzyme product observed in the presence of Cys (Fig. 5A). Cys also drove a faster forward E2P dephosphorylation that would account for the higher enzyme turnover in the presence of the amino acid. Although, it could be argued that the faster dephosphorylation might be driven by the

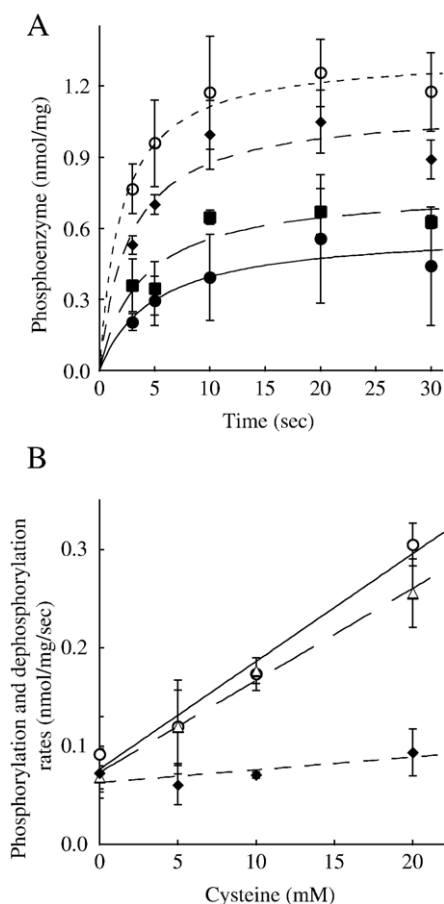


Fig. 5. Effect of Cys on CopA phosphorylation and dephosphorylation reaction. (A) Phosphorylation of CopA by ATP was measured in the presence of various Cys concentrations: 0 mM (●), 2 mM (■), 10 mM (◆), 20 mM (○). (B) Phosphorylation and dephosphorylation rates were measured at the indicated Cys concentration and calculated assuming a constant rate during the first 3 s of reaction. Phosphorylation rates (△), E2P dephosphorylation rates (○), E1P dephosphorylation rates (●). Points are the mean±SE of three independent experiments.

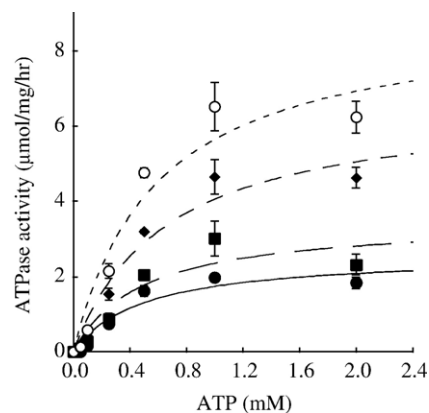


Fig. 6. Effect of Cys on CopA activation by ATP. ATPase activity was measured in the presence of various Cys concentrations and indicated levels of ATP. Curves were fitted using the V_{max} (μmol/mg/h) reported in Fig. 1 and K_m (mM): 0.49 ± 0.14 , 0 mM Cys (●); 0.48 ± 0.18 , 2 mM Cys (■); 0.65 ± 0.14 , 10 mM Cys (◆); 0.58 ± 0.15 , 20 mM Cys (○). Points are the mean±SE of three independent experiments.

higher phosphoenzyme level, a direct effect of Cys on the E2P form of the enzyme cannot be disregarded. Finally, while Cys appears to affect the transitions associated with the production and hydrolysis of phosphoenzyme forms of CopA, it did not appear to alter the $E1 \leftrightarrow E2$ equilibrium. In this direction, the low affinity ATP activation of CopA ATPase activity was not affected by Cys (Fig. 6).

4. Discussion

Mechanistic models of alkali metal transmembrane transport are based on the interaction of the free (hydrated) ions with the protein transporters. On the contrary, Cu^+ does not appear to be “free” in biological systems [39]. Thus, the development of a mechanistic depiction of Cu^+ transport, by P-type ATPases in our case, requires the interaction of a chelated or protein-bound metal with the transmembrane protein. This would be followed by a metal ligand exchange where the metal is delivered to the transport site. This process is distinct and independent of Cu^+ binding to a regulatory cytoplasmic site. These mechanistic requirements associated with Cu^+ access to transmembrane transport sites have not been described either kinetically or structurally. Consequently, it becomes relevant to identify and understand parsimonious systems that, although non-physiological, might mimic *in vivo* conditions. Such experimental tool might be provided by the stimulatory effect of Cys on the transport and ATPase activity of some P_{IB} -type ATPases. To explore this possibility, we characterized the effect of Cys on *A. fulgidus* CopA.

4.1. Mechanisms of CopA activation by Cys

The presence of millimolar concentrations of Cys activates *A. fulgidus* CopA. This appears independent of the presence of Cu^+ . First, Cys $K_{1/2}$ for the activating effect is three orders of magnitude larger than Cu^+ (or Cu^+ -thiolate) $K_{1/2}$ for enzyme

activation. Keeping in mind the high association constant for the Cys–Cu⁺ complex [30], it is clear that Cys alone (not Cys–Cu⁺) has an activating effect. Secondly, in the absence of metals Cys stimulates CopA pNPPase activity in fashion similar to its ATPase activity. Consistent with a metal independent activation occurring in the presence of saturating Cys–Cu⁺, it can be assumed that Cys activates the enzyme by interacting in an allosteric fashion. This allosteric site would be located in the cytoplasmic side of CopA, since it is the presence of Cys on this side rather than on the extracellular face, which activates the enzyme. However, Cys–protein interaction does not seem to involve the N-MBD because Cys stimulation of T-CopA is identical to that of CopA. Lastly, this site is accessible when the enzyme is in the E1 conformation as evidenced by the activation of the phosphorylation reaction under non-turnover conditions.

Cys interaction with CopA leads to higher phosphorylation and dephosphorylation rates along with higher steady state levels of phosphoenzyme intermediary. While the direct effect on the phosphorylation step can be assumed, whether the faster dephosphorylation is only the result of the higher phosphoprotein level or also include a direct effect of Cys cannot be discriminated. This is because of the errors associated with our manual assays, the relatively long time of our first measurement point, and a reaction kinetics that might not be a simple exponential. To resolve this question is beyond the scope of this report. Nevertheless, the data indicate that Cys affects the enzyme metal release and dephosphorylation. Since these appear as rate limiting steps in the CopA reaction cycle [16], it is logic to conclude that the more rapid dephosphorylation leads to the observed faster enzyme turnover.

4.2. Mechanistic implications of Cys–Cu⁺ dependent ATPase activity

Considering the conditions of our experiments, even at the higher tested Cu⁺/Cys ratio the concentration of free Cu⁺ was under 10^{−9} M. These Cu⁺ levels cannot activate the enzyme ($K_{1/2} = 2.1 \pm 0.6 \mu\text{M}$ Cu⁺ in the absence of Cys). Then, to explain the experimental observations it is necessary to postulate that Cys–Cu⁺ “delivers” Cu⁺ to the protein. Where in the enzyme does the Cu⁺ exchange between Cys (or other ligands) and the protein occur? This “delivery” does not involve the N-MBD, since the truncated protein responds to Cys–Cu⁺ as the wild type CopA. It could be hypothesized that Cu⁺ ligand exchange occurs at the transmembrane binding site formed by residues from H6, H7, and H8 [2]. The structure of the related SERCA1 Ca-ATPase shows that Ca²⁺ access to the transmembrane binding sites is through a relatively wide (8–10 Å) funnel that contains one of the Ca²⁺ ligands (equivalent to the proximal Cys in CopA H6) at its bottom [40]. If a similar wide shallow funnel were present in CopA, Cu⁺ might be “delivered” directly into the transmembrane metal binding site.

In summary, this report shows that Cys allosterically activates *A. fulgidus* CopA and that Cu⁺-ATPases might receive the bound metal directly at the transmembrane transport site.

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