CopB from *Archaeoglobus fulgidus*: a thermophilic Cu^{2+} transporting CPx-ATPase

by

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ABSTRACT

In this work we present the first characterization of a Cu^{2+}-transporting ATPase. The thermophilic bacteria *Archaeoglobus fulgidus* contains two genes, CopA and CopB, encoding for CPx-ATPases. CopB belongs to the subgroup IB-4 of the CPX-ATPases. These enzymes are characterized by a CPH motif in the 6th transmembrane domain and a His-rich N-terminus metal binding domain (MBD). CopB was heterologously expressed in *E. coli*. Membranes were prepared and used to measure activity. CopB was active at high temperature (75º C), high ionic strength and pH 5.7. The enzyme was activated by Cu^{2+}, and in to a lesser extent by Ag^{+} and Cu^{+}. CopB showed a V_{max} = 5 μmol/mg/h and a high apparent affinity ($K_{1/2}$ = 0.28 ± 0.09 μM) for Cu^{2+}. Uptake of $^{64}Cu^{2+}$ into everted vesicles was also measured in order to show that Cu^{2+} is not only activating the enzyme but being transported. Compared with CopB-WT, CopB-T (lacking the N-terminus MBD) did not show any difference in its activation by the different metal ions, demonstrating that the cytoplasmic MBD has no role in the metal selectivity. CopB-T also showed a 40 % decrease in the ATPase activity. CopB-WT and CopB-T presented similar levels of phosphorylation. However, CopB-T exhibited a reduced rate of dephosphorylation (slower transition from the E_2P to the E_2 conformation). These observations suggest a regulatory role for the cytoplasmic MBD.

Key Words: CPx-ATPase, CopB, Cu^{2+}, His-rich

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LIST OF ABREVIATIONS

BCA: Bicinchoninic acid

BCS: Bathocuproindisulfonate

CBB: Coomassie brilliant blue

DTT: Dithiothreitol

IC_{50}: concentration of inhibitor required for a 50% decrease in enzyme activity

MBD: Metal binding domain

MNK: Menkes protein

Pi: Inorganic phosphate

SDS: Sodium dodecil sulfate

SOD: Super oxide dismutase

TTM: Tetrathiomolybdate

WND: Wilson protein

1. INTRODUCTION

1.1. Metals and life

Metal ions play many different roles within cells. Metals such as Mg, Fe, Cu, and Zn act as cofactors in enzyme reactions including group transfer, redox and hydrolysis. (Fraústo da Silva and Williams, 2001) Others like Na, K, and Ca are involved in physiological processes and/or maintaining structure as well as controlling the function of cell walls. It has been estimated that over a quarter of all known enzymes require a particular metal ion for function. These enzymes can be divided into two groups: metal-activated enzymes and metalloenzymes. Metal-activated enzymes require the addition of metal ions to become stimulated (Horton *et al.,* 2002). Kinases are an example of this type of enzymes because of its use of the Mg-ATP complex as a phosphoryl group-donating substrate. Metalloenzymes necessitate metal ions to function properly. The metal ion is firmly bound to the enzyme and is frequently recycled after protein degradation. Heme groups in hemoglobin or cytochromes bind a Fe^{2+} ion tightly. The Chlorophyll that sustains every ecosystem also needs a Mg^{2+} ion. The Cu-Zn super oxide dismutases (SOD) require metal ions to detoxify the cell from dangerous free radicals. These are some of the many examples of diverse roles that metal ions play.

Metal ions are essential for life, yet they are toxic to the cells at high concentrations or in the free form (Fraústo da Silva and Williams, 2001; Hughes, 1981). They can generate free radicals, which are highly reactive and oxidant molecules that can react with any biomolecule such as nucleic acids. This interaction with biomolecules may

eventually lead to cell death. Cells have developed highly complex systems used for sensing, trafficking and the transport of metals. Many of these systems are not well understood yet (Outten and O'Halloran, 2001; Huffman and O'Halloran, 2000; Gatti *et al.,* 2000; Harrison *et al.*, 2000; Rensing *et al.*, 1999).

1.2. Thermophilic proteins

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. Members of the Crenarqueota kingdom of archaea are known to grow at temperatures higher than 80° C. These organisms called "hyperthermophilic" are able to live because of the stability of their biomolecules (Madigan *et al.*, 2000).

It has been known for some time that several thermophilic enzymes exhibit a significant half-life at the boiling point of water (Coolbear *et al.*, 1992). Different structural studies have been done comparing these proteins with their counterparts in the mesophilic world (Russell *et al.*, 1998; Auerbach *et al.*, 1998). The main finding was that there is not a "single pattern" in the structural differences that confers the protein's stability at high temperatures. For a given group of proteins some characteristics of thermophilic proteins can be identified, but these structural differences can differ for another group. Moreover, changes in even a few residues showed significant changes in thermal stability (Daniel and Cowan, 2000)

1.3. P-type ATPases

Homeostasis and extrusion of solutes were processes developed early by the cells in order to survive (Rensing *et al.*, 1999a). The plasma membrane is the essential permeability barrier that separates the inside of the cell from the outside. However, it should also allow the transport of desired solutes. Transmembrane transport proteins are ubiquitous proteins that regulate intracellular concentrations of solutes by either extrusion or accumulation in sub-cellular organelles. P-Type ATPases are a ubiquitous family of transmembrane proteins involved in the cation transport against electrochemical gradients. The main structural characteristics of these enzymes are six to ten transmembrane α-helices (H1-H10) and one ATP binding domain. A highly conserved sequence (DKTGT) is present in the large cytoplasmic loop, and phosphorylation of the aspartic acid residue drives the key conformational changes in the protein (Lutsenko and Kaplan, 1995).

The P-type ATPase is an extensive and expanding family of proteins that is divided into five groups (and some sub-groups) based on sequence alignments and putative ion specificity (Axelsen and Palmgren, 1998). In this way, the group P_1 is divided into: a: K^+ and b: heavy metals (Cu^+, Ag^+, Cu^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+}) transporters. Group P_2: a: Ca^{2+}, Mn^{2+} including SERCA pumps; b: Ca^{2+}; including PMCA pumps; c: Na^+/K^+; H^+/K^+ and d: possible Na^+; Ca^{2+} pumps. Group P_3: a: H^+ and b: Mg^{2+} transporters. P_4 and P_5 are groups with unknown selectivity but it was proposed that P_4 could be related to the transport of lipids. In Fig 1 the different groups of P-type ATPases are represented. In

this phylogenetic analysis, the authors show the diversity that these transporters present and how "divergent" the groups are in relation to a proposed ancestor.

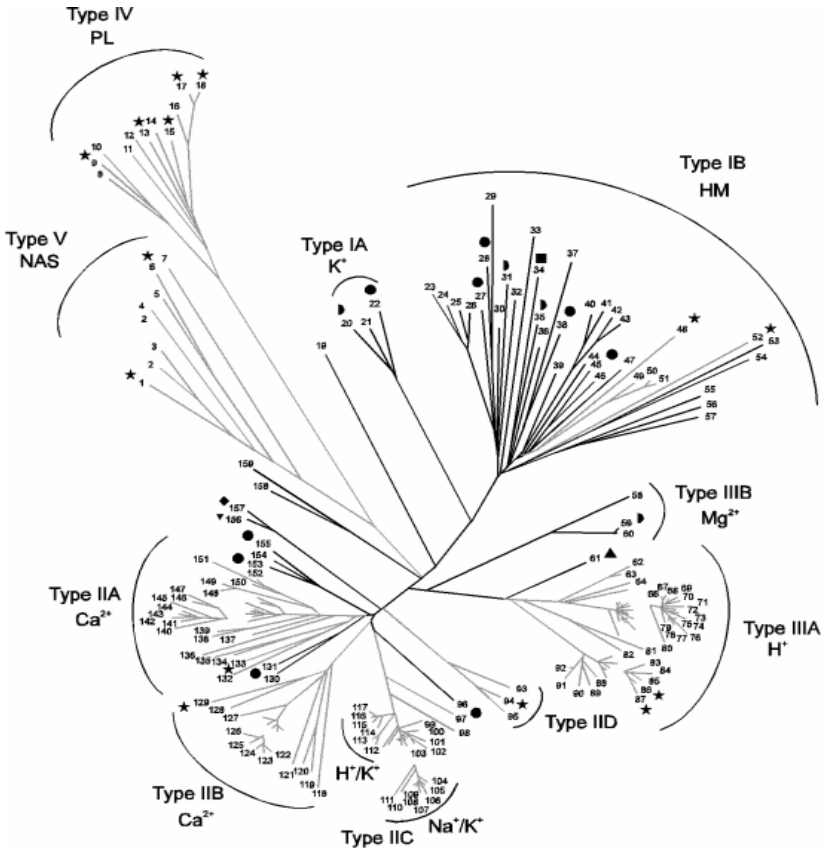

Fig 1: Phylogenic tree of P-type ATPases (taken from Axelsen and Palmgren, 1998)

Recently, the sarcoplasmic reticulum Ca ATPases has been crystallized despite the constraints for crystallization of membranous proteins (Toyoshima *et al.*, 2000). This report represent a big step in the understanding of the domains involved in the coordination of the metal ion and the conformational changes of the enzyme towards ion binding.

1.4. Heavy metal ATPases

Heavy metal ATPases, also called CPx-ATPases, belong to the P_{1B} group of the P-type ATPases. Possibly the better studied enzymes of this subfamily are the two human Cu^{+} transporters ATP7A and ATP7B or Menkes (MNK) and Wilson (WND) Disease proteins, respectively. Like many human proteins, they are known by the name of the disease they can cause through mutations in their respective genes. MNK is found in the plasma membrane and mutations in this protein cause systemic Cu deficiency (Bull *et al.*, 1993). WND is expressed in the liver and its mutations result in systemic overload of Cu from the lack of hepatic clearance (Petrukhin *et al.*, 1994)

CPx-ATPases are composed of eight transmembrane domains, an ATP binding site in the cytoplasmic loop between H6 and H7, and the consensus sequence "CPx" in the sixth transmembrane domain (Solioz and Vulple, 1996). The CPx motif has been demonstrated to be essential for the translocation of the metal ion (Fan and Rosen, 2002; Yoshimizu *et al.*, 1998). Cytoplasmic metal binding domains (MBDs) are also a characteristic of most of these transporters. MBDs are situated in the cytoplasmic N-terminus region of the enzymes. These domains are mainly characterized by a His-rich motif or the consensus sequence CxxC, which is also present in some metal chaperones (Arnesano *et al.*, 2002). CxxC MBDs can be found as single motif (bacteria) or multiple (yeast, human) (Voskovoinik *et al.*, 1999). Strikingly, it was demonstrated that for different CPx-ATPases these motifs are not essential for enzyme activity (Voskovoinik *et al.*, 1999; Mitra *et al.,* 1999; Argüello *et al.*, 2003) or for the metal ion selectivity process (Mitra and Sharma, 2001; Argüello *et al.*, 2003). However, Tsivkovskii *et al.,* (2001) have

shown that the CxxC motif interacts with the ATP binding domain in a Cu^{+} dependent manner. Cu^{+}-bound MBD diminishes its interaction with the ATP binding domain, which suggests a regulatory role for this particular MBD.

Experimental evidence suggests that CPx-ATPases operate in a catalytic cycle similar to all P-type ATPases, changing conformation between states E1 and E2 (Okkeri *et al.*, 2002; Mandal *et al.*, 2002). The catalytic cycle of CPx-ATPases is represented in Fig. 2. This cycle is characterized by the coupled reaction of ion translocation and ATP hydrolysis (Glynn, 1985). The terminal phosphate group of ATP (complexed with Mg^{2+}) is transferred to the conserved aspartyl residue in the DKTGT sequence. The enzyme then translocates the metal ion through the membrane, changing from the E_1P state to E_2P state. Finally the phosphate group is hydrolyzed and the enzyme returns to it original state.

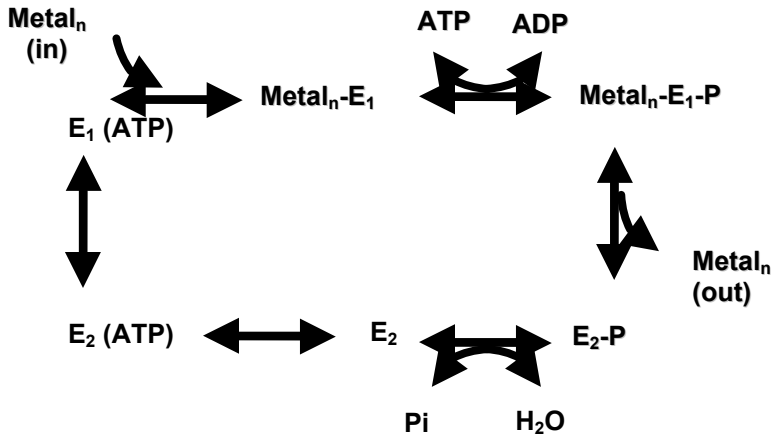

Fig 2: Scheme of the catalytic cycle of CPx-ATPases. The metal ion is translocated using the energy of the ATP to change the conformational state of the enzyme.

1.5. His-rich ATPases

Much less work has been reported on His-rich CPx-ATPases and the overall characteristics of these enzymes remain unclear (Tong, *et al.*, 2002; Wyler-Duda and Solioz, 1996; Solioz and Odermatt, 1995). *A. fulgidus* CopB ortholog in *Enterococcus hirae* is the only the IB-4 CPx-ATPases that was partially characterized (Bissi *et al*., 2001; Wyler-Duda and Solioz, 1996; Solioz and Odermatt, 1995; Odermatt *et al.*, 1994; Odermatt *et al*., 1993). This enzyme was cloned as part of the CopAB operon (Odermatt *et al*., 1993). It was shown the expression by induction with different metals and resistance to copper and silver by measuring growth rate of the WT or ΔCopB cells in presence of the different ions. They found that the expression of CopB was been induce by $CuSO_4$, $AgNO_3$ and $CdCl_2$. *E. hirae* ΔCopB cells were also unable to grow in $CuSO_4$ supplemented media even the presence of a functional CopA. This is notable because CopA ortholog in *A. fulgidus* was reported as Cu^+ extrusion pump, though conferring resistance to copper in oxidation state (I) (Mandal *et al.*, 2002). Further work in ΔCopB cells demonstrated that this cells bioaccumulated five fold more copper than the WT (Odermatt *et al.*, 1994). Uptake of ^{64}Cu and ^{110}Ag into everted vesicles at pH 6, showed V_{max} = 4.2 nmol/mg/h towards both metal ions and $K_{1/2}$ = 1μM for Cu^+ and Ag^+. (Solioz and Odermatt, 1995). The CopB purification to homogeneity and further reconstitution into phospholipid vesicles was attempted, but hydrolysis of ATP stimulated by Cu^+ or Ag^+ could not be measured (Wyler-Duda and Solioz, 1996). The inhibition of the CopB ATPase activity was determined for different compounds, BCA, BCS, DTT and TTM with IC_{50} values of 14 μM, 4 μM, 3 μM and 34 nM, respectively (Bissi *et al*., 2001). *E.*

hirae CopB was also used as a model to study the mutations responsible of MNK and WDP diseases (Bissig *et al.*, 2001). A common mutation in the transmembrane MBD of MNK was mimicked in CopB. The mutant enzyme failed to complement ΔCopB cells growing in copper supplemented media. The ATPase activity of CopB-WT showed a Vmax = 1.4 ± 0.31 μmol/mg/min and the mutant presented no ATPase activity. *E. hirae* CopB has been reported as a Cu^{+}/Ag^{+} ATPase in spite of imidazolium ligands at both MBDs that should have more affinity for Cu^{2+} than Cu^{+} (Fraústo da Silva and Williams, 2001; Hughes, 1981). Moreover the interaction between Cu^{2+} and N at imidazole rings present in N-terminus His-rich domains have been reported for different proteins (Brewer and Lajoie, 2000; Battistoni *et al.*, 2001; Hashimoto *et al.*, 1998).

1.6. CPx-ATPases in *Archaeoglobus fulgidus*

The thermophilic archea *Archaeoglobus fulgidus* presents two CPx ATPases, CopA and CopB. CopA belong to the subgroup IB-1 of the CPx-ATPases. This subgroup is characterized by a CPC motif in H6 and the CxxC signature as cytoplasmic MBD. Our group has already characterized this enzyme as a Cu^{+}/Ag^{+} ATPase (Mandal *et al*., 2002)., CopA was heterologously expressed in *E. coli* as a fusion protein with a hexahistidine tag and purified to homogeneity using a Ni^{2+} affinity column. The lipidic environment was provided by phosphatydil choline-enriched lipids. The enzyme was active at 75° C, 400 mM NaCl, pH 6.1 and 20 mM cysteine. The ATPase activity of CopA was stimulated by the monovalent cations Cu^{+} (V_{max} = 3.66 μmol/mg/h and a $K_{1/2}$ = 2.1 μM) and in a higher extent by Ag^{+} (V_{max} = 14.82 μmol/mg/h and a $K_{1/2}$ = 29.4 μM). The enzyme also showed

interaction with ATP with two apparent affinities (ATPase $K_{1/2}$ = 0.25 mM and phosphorylation Km = 4.81 μM). The phosphoenzyme levels in presence of both metal ions were measured using [γ-^{32}P] ATP, showing similar levels of phosphorylation. It is a characteristic of CPx-ATPases to be inhibited by vanadate. The ATPase activity of CopA was inhibited with an IC_{50} = 24 μM.

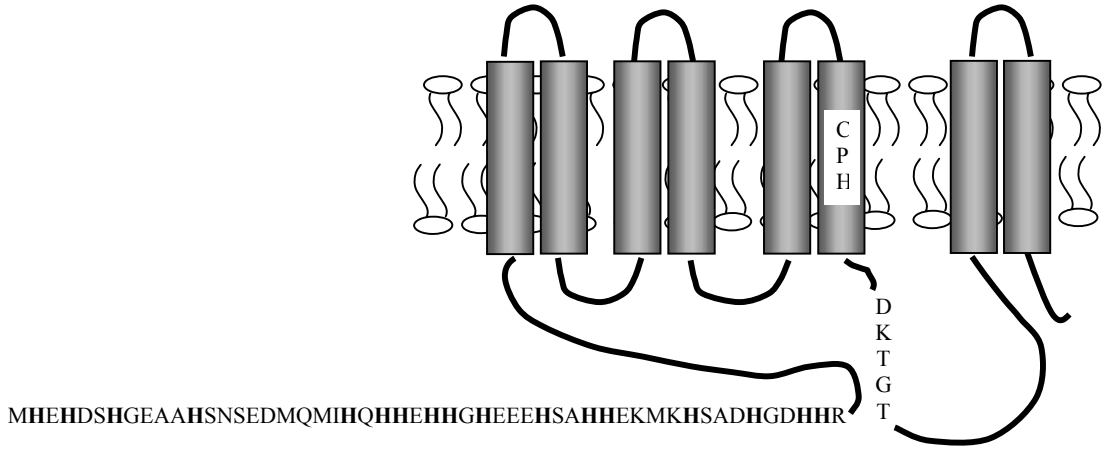

Fig 3: Membrane topology scheme of CopB as predicted by hydrophobicity plots. The N-terminus cytoplasmic MBD presenting the 17 His residues and the CPx-ATPases conserved sequences "CPH" and "DKTGT" are also shown.

On the other hand, CopB belong to the subgroup P1B-4 of the CPx-ATPases. CopB presents His residues at both MBDs; a His-rich motif (which includes seventeen single or double His residues) as cytoplasmic N-terminal MBD and the "CPH" motif in the H6 (Fig 3).

In this work we report the heterologous expression as well as the functional and ion selectivity characterization of CopB from *A. fulgidus*. Here, we present experimental evidence of CopB as a Cu^{2+} CPx-ATPase. We were also able of demonstrate that the His-

rich domain of CopB is not involved in the selectivity of the metal ion. Moreover, truncation of the enzyme leads to a slower rate of dephosphorylation, suggesting a regulatory role for this N-terminus domain. This is the first characterization of Cu^{2+}-ATPase.

2. METHODS

2.1. ***Cloning and expression***

The gene AF0152 from *A. fulgidus* encoding for the CPx-ATPase CopB cloned into pBADTOPO/His vector (Invitrogen, Carlsbad, CA) was used in this study. The gene sequence was confirmed by automated DNA sequence analysis. This vector introduces a carboxyl terminal hexahistidine tag suitable for immuno-detection and purification. A second construct containing the CopB-T form of CopB (lacking the N-terminus 51 amino acids) was done by polymerase chain reaction (Expand, Roche) using the primers 5′-ATGGAGGACTTCAAGAAGCGATTCTA-3′ and 5′- CCGCAGCAATCTGGCATTA ATGGCCAC-3′. The resulting fragment was subcloned in pBADTOPO/ His vector vector. *E. coli* Top10 were co-transformed with the respective constructs and the vector pSJS1240 encoding for rare tRNAs (tRNA argAGA/AGG and tRNA ileAUA) (Kim *et al.,* 1998). Cells were grown at 37ºC in 2 x YT media supplemented with 150 μg/ml of ampicillin and 50 μg/ml chloroanphenicol. Protein expression was induced with 0.002% L-arabinose. Cells were harvested after 3 h of induction, washed with 25 mM Tris, pH 7.0, 100 mM KCl and stored at -70°C.

2.2. ***Membrane preparation***

Membranes were obtained in accordance with Mandal *et al*., (2002). Briefly, cells were suspended in buffer A (25 mM Tris, pH 7.0, 100 mM sucrose, 1 mM phenyl methyl

sulfonyl fluoride) and disrupted in a French Press at 20,000 psi. After the addition of 0.02 mg/ml Dnase I and 2 mM $MgCl_2$, the homogenate was incubated for 30 min at 4°C. Lysed cells were then centrifuged at 9,000 g for 30 min. The supernatant was centrifuged at 163,000 g at 4°C for 1 h, washed with buffer A, re-centrifuged and finally resuspended in buffer A at a protein concentration of 10-15 mg/ml. The membranes were stored at -70°C. All protein determinations were performed in accordance with Bradford. 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 (CBB) and immunobloting using anti-His_6 antibody (Invitrogen, Carlsbad, CA).

2.3. ***ATPase assays***

The ATPase assay mixture contained 50 mM Tris-Cl, pH (75º C) 5.7, 3 mM $MgCl_2$, 3 mM ATP, 400 mM NaCl and membranes at a concentration of 0.02 mg/ml. dithiothreitol (DTT), bicinchoninic acid (BCA), Cysteine and the different metals were independently varied as indicated in the corresponding figures. The buffer was prepared at room temperature and the pH at 75 ºC was calculated using pKa/ºC conversion factors (Tris: -0.031). ATPase activity was measured after 10 min incubation at 75º C. Hydrolyzed inorganic phosphate (Pi) was determined in accordance with Lanzetta *et al.,* (1979).

2.4. Everted vesicles preparation

Vesicle preparation was done in accordance with Tsai *et al.,* (1992). Briefly, washed cells (see above) were resuspended in sucrose buffer (50 mM MOPS-KOH pH 7.0, 250 mM sucrose, 200 mM KCl and 10 mM $MgSO_4$) at a concentration of 5 mg/ml. Lysozyme (1 mg/ml) was added and the cell suspension was incubated on a shaker (200 RPM) at 37º C for 1 h. Protoplasts were lysed in a French Press at 10,000 psi and the everted membranes were separated from the cell debris by two sequential centrifugations at 12,000 g for 1h. The membranes were pelleted at 160, 000 g and resuspended in sucrose buffer.

2.5. ^{64}Cu transport assays

Cu^{2+} uptake into everted vesicles was measured using $^{64}Cu^{2+}$ (Washington University, Medical School, Mallinckrodt Institute of Radiology, NCI Research Resource Grant 1R24 CA 86307). Membrane vesicles (1 mg/ml) were incubated in 50 mM Bis-Tris pH (55º C) 5.7, 3 mM $MgCl_2$, 400 mM NaCl and 5 µM $CuSO_4$ (0.6 µCi/ml) at 55º C. After 1 min, the reaction was started by the addition of 3 mM ATP. Aliquots were then transferred at the specified times to 0.2 µm cellulose nitrate filters (Millipore Corp., Bedford, MA). The filters were washed with 15 ml of ice-chilled 25 mM Tris-Cl pH 7.0, 0.2 M KCl, 0.25 M sucrose, 10 mM $MgSO_4$ and 2 mM $CuSO_4$. Radioactivity was measured in a scintillation counter.

2.6. Phosphorylation Assays

Enzyme phosphorylation by ATP was carried out in a medium containing 50 mM bis Tris pH (37º C) 5.7, 1 mM $MgCl_2$, 1 μM [γ-^{32}P] ATP, 400 mM NaCl, 20 % dimethyl sulfoxide, 1 mg/ml of membranes and 1 μM $CuSO_4$. 2.5 mM DTT was added when required. The reaction was initiated by the addition of [γ-^{32}P] ATP and stopped after 30 s at 37°C with 5 volumes of ice-cold 10% trichloroacetic acid, 1 mM $NaPO_4$. The samples were then centrifuged at 14,000 g for 10 min, washed with ice cold water and resuspended in acidic gel loading buffer (5 mM Tris-PO_4, pH (20º C) 5.8, 6.7 M Urea, 0.4 M DTT, 5% SDS and 0.014% Bromo Phenol Blue). Afterwards, the samples were loaded in 8% acidic polyacrylamide gel as described in Sarkardi *et al.,* (1985). The gels were dried, exposed to imaging plates and then analyzed in a phosphoimager.

2.7. Dephosphorylation Assays

The time course of enzyme dephosphorylation was examined for CopB-WT and CopB-T. Phosphorylation was carried out as described above. EDTA at a concentration of 5 mM was added to the medium after 30 s instead of the acid stopping solution and the samples were incubated for another 3, 6, 9 or 12 s at 37 ºC. The incubations were stopped with 10 % trichloroacetic acid containing 1 mM $NaPO_4$. Samples were then centrifuged and loaded in 8% acidic polyacrylamide gels as described above.

2.8. Data Analysis

Curves of ATPase activity versus Cu^{2+}, Cu^{+} or ATP were fit to $v=V_{max}\ L/(L+K_{1/2})$, where L is the concentration of the variable ligand. Data analysis was performed using the KaleidaGraph software (Synergy, Reading, PA). Experimental values are the mean ± S.E. of at least two independent experiments performed in duplicate. The reported standard errors for V_{max}, $K_{1/2}$, and E^{a} are asymptotic standard errors reported by the fitting program. Sequence analysis and percent identity (Clustal V method) were performed using LaserGene Software (DNASTAR, Madison, WI).

3. RESULTS

A. fulgidus is a hyperthermophilic archaea that has two CPx-ATPases encoding genes, AF0473 (CopA) and AF0152 (CopB) (Klenk *et al*., 1997). Both ATPases are related to the group of $Cu^{+}/Cu^{++}/Ag^{+}$ transporting-ATPases and show 36.1% of sequence homology. *A. fulgidus* CopA has a CxxC-like N-terminus MBD and a CPC metal binding motif in the 6th transmembrane domain. It was also reported to be a Cu^{+}/Ag^{+} extrusion pump (Mandal *et al.*, 2002). On the other hand, CopB from *A. fulgidus* has a His-rich N-terminus MBD, a CPH metal binding motif in H6 (Fig 3) and 41.6% of sequence homology with the *E. hirae* CopB. Fig 4 shows an alignment of different CPx-ATPases showing the conserved motifs "CPx" and DKTGT.

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TCPHALGLAIPLVVAVSTSLAAKSGLLIRDRQAFERAKDLQAVIFDKTGTL CopB A. fulgidus
ACPHALGLAIPLVVARSTSIAAKNGLLLKNRNAMEQANDLDVIMLDKTGTL CopB E.hirae
SCPCGLVISIPLGYFGGIGGAAKRGILVKGSTFLDTLTAVKTVVLDKTGTL BXA1 O. brevis
ACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKVKVVVFDKTGTI MNK  H. sapiens
ACPCAFGLATPTALTVGMGKGAELGILIKNADALEVAEKVTAVIFDKTGTL CopA A. fulgidus
ACPCALGLATPTAVMVATGVGATNGVLIKGGDALEKAHKVKYVIFDKTGTL RAN1 A. thaliana
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Fig 4: Alignment of different P_{1B} ATPases related to copper transport. In grey background can be seen the CPx motif in H6 and the DKTGT conserved sequence, where the aspartic acid residue is phosphorylated. Bacterial CPx-ATPases: CopB A.fulgidus (O30085), CopA A.fulgidus (O29777), CopB E.hirae (P05425) and Bxa1 (AB073990). Plant CPx-ATPase: RAN1 (Q9S7J8). Human CPx-ATPase: MNK (Q04656). The alignment was performed by Clustal V method using LaserGene Software (DNASTAR, Madison, WI).

3.1. Subcloning of CopB-T in pBADTOPO vector

A cDNA clone of CopB from *A. fulgidus* in a pBAD TOPO vector available in our lab was used in these studies. The identity of this clone was verified by automated sequencing. In order to evaluate the functional role of the N-terminus MBD, a mutant lacking this domain was amplified by polymerase chain reaction using pBADTOPO/CopB-WT as template. The cDNA was sub-cloned into the same vector. The mutant sequence was corroborated by automated sequencing. Fig 5 shows the cDNA amplification of CopB-WT (2070 bp) and CopB-T (1965 bp).

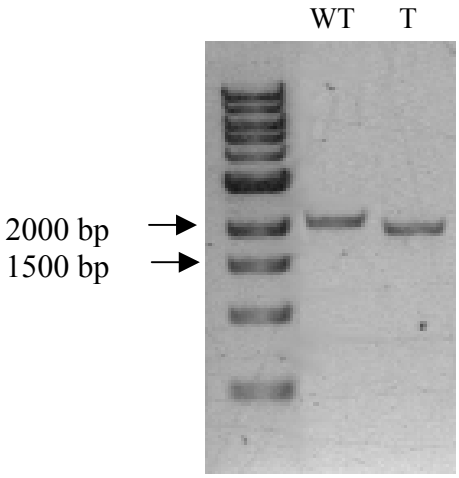

Fig 5: DNA gel of CopB-WT and CopB-T cDNA. PCR amplifications of the different clones were done using plasmidic DNA as a template and loaded in a 1% agarose gel containing ethidium bromine.

3.2. Expression of CopB-WT and CopB-T

The CopB-WT and CopB-T were expressed in *E. coli* TOP10 carrying an extra plasmid encoding for rare tRNAs (see Methods). The expressed proteins were only detected in the membrane fraction (data not shown). Fig 6 shows the presence of CopB-WT and CopB-T in the membrane fraction obtained from the corresponding cells after induction with arabinose. Amounts of 35 to 50 mg of membranes were usually obtained per liter of culture.

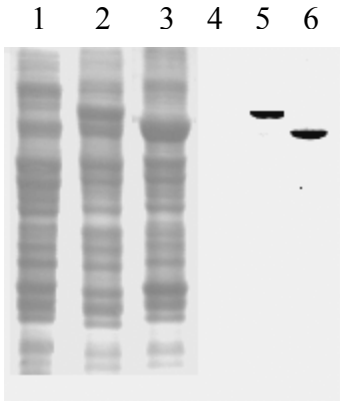

Fig 6: Expression of CopB-WT and the CopB-T mutant from *A. fulgidus.* Lanes 1and 4: untransformed *E. coli*; lanes 2 and 5: arabinose induced *E. coli* transformed with pBADTOPO/ CopB-WT; lanes 3 and 6: arabinose induced *E. coli* transformed with pBADTOPO/CopB-T. Lanes 1-3: 10 % SDS-PAGE stained with coomassie brilliant blue; lanes 4-6 immunoblot with anti-His antibody.

3.3. ATPase activity assays

The first goal was to get the optimal conditions for measuring the activity of CopB. The ATPase activity was measured under the below cited variables. CopB was stimulated by Cu^{2+} at high temperatures (Fig 7). The enzyme was inactive at 40º C and reached a maximum of ATPase activity at 75º C. This characteristic is not surprising for a protein from hyperthermophilic archea with an optimum growth temperature of 83º C (Madigan *et al.*, 2000). However, the steep decrease in activity after the optimum temperature is probably related to experimental limitations of working with mesophilic membranes.

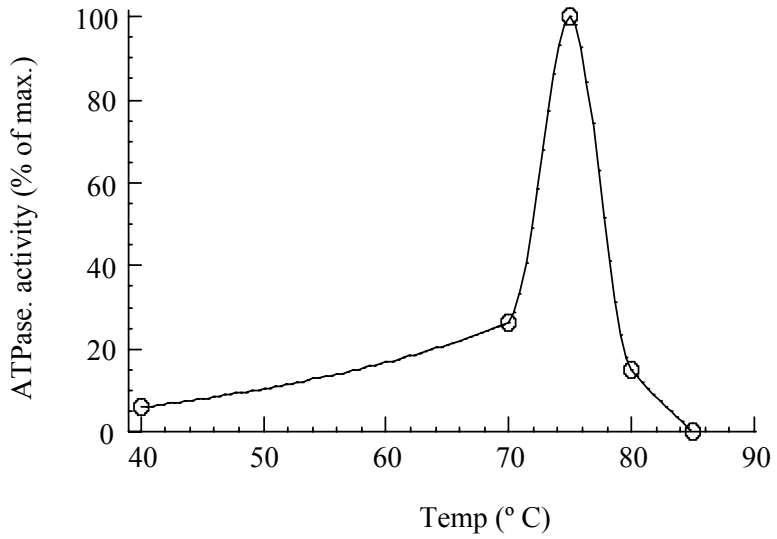

Fig 7: Dependence of CopB ATPase activity on the temperature. ATPase activity was determined at different temperatures in the presence of 1μM $CuSO_4$. The ATPase activity was measured at pH 5.7, 400 mM NaCl and 1 μM Cu^{2+}.

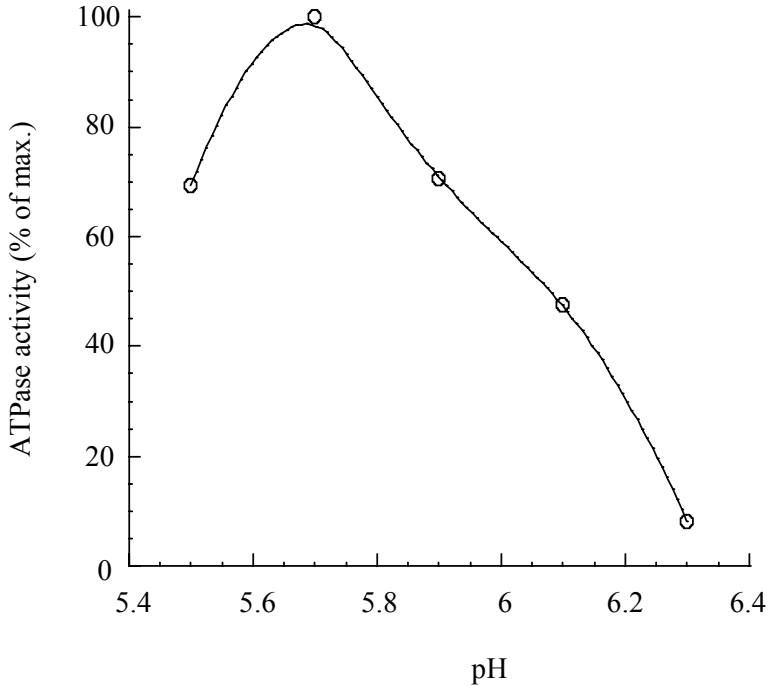

Fig 8: Dependence of CopB ATPase activity on pH. The ATPase activity was measured at different pH values using Tris-Cl at 75° C, in the presence of 400 mM NaCl and 1 μM Cu^{2+}.

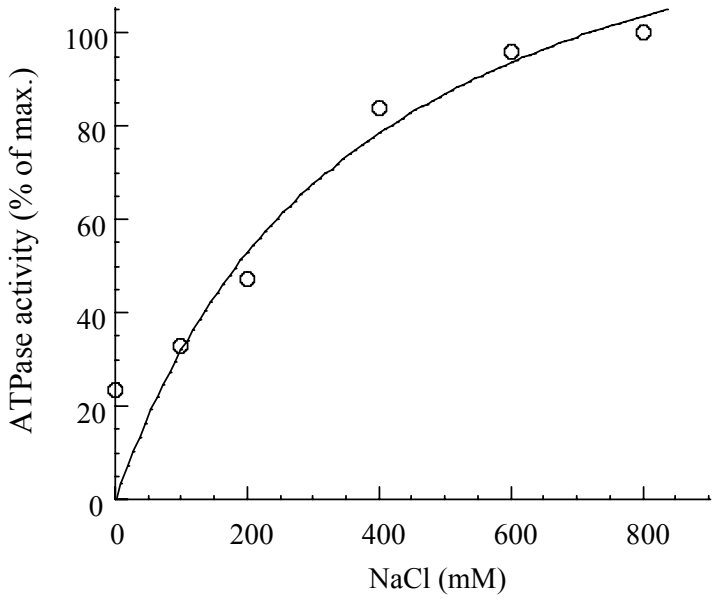

Fig 9: Dependence of CopB ATPase activity on ionic strength. The ATPase activity was measured at different concentrations of NaCl at 75° C, pH 5.7 and the presence of 1 μM Cu^{2+}.

We also wanted to determine the optimum pH at which the ATPase is activated by Cu^{2+}. CopB showed typical bell-shaped pH dependence, having a maximum of ATPase activity at pH 5.7 (Fig 8). The ATPase activity was also stimulated at high ionic strength reaching a plateau after 400 mM NaCl (Fig 9). The addition of 400 mM NaCl increased four fold the ATPase activity. CopB was shown to be active into the phospholipid bi-layer membrane of mesophilic bacteria at an optimum temperature of 75º C, pH 5.7and 400 mM NaCl

3.4. Ion selectivity of the CopB-WT and CopB-T

Our first question concerning to the functional characterization was to determine which metals activate CopB. The ATPase activity of CopB-WT was measured upon activation with different metals. CopB-T was also assayed with the purpose of knowing if the ability of the enzyme to recognize a particular metal ion to transport was residing in the cytoplasmic MBD. Results are shown a percentage of the maximum value in Fig 10. CopB was activated by $Cu^{2+}>>Ag^{+}>Cu^{+}$. Cu^{2+} stimulated ATPase activity of activated CopB approximately 40% higher than Ag^{+} and 70% more than Cu^{+}. CopB-WT and CopB-T were activated to the same relative extent by the same metals demonstrating that the cytoplasmic MBD has no role in metal ion selectivity.

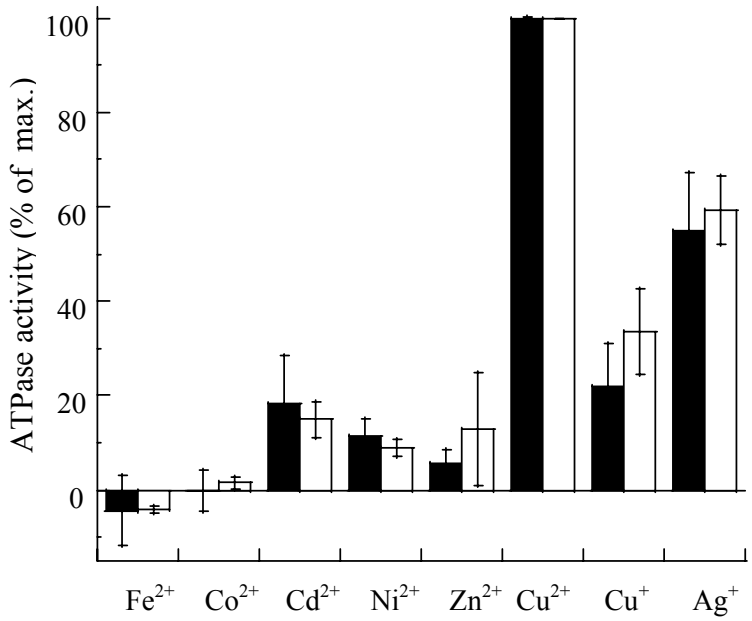

Fig 10: Activation of CopB-WT (■) and CopB-T (□) by different metals. ATPase activities of CopB-WT and CopB-T were taken at 1 μM concentration of the indicated metal. In the Cu^+ containing assay mixture 2.5 mM DTT was also included. 100% = 5.0 ± 0.3 μmol/mg/h for CopB-WT and 3.0 ± 0.3 μmol/mg/h for CopB-T.

Different concentrations of the each metal were tested to determine the concentration at which the enzyme reaches V_{max} for this particular ion. Metals that activate CopB did not show further activation over 1 μM and the metals that did not activate it show the same result at higher concentrations.

3.5. Copper activation of the CopB ATPase activity

The Cu^+ chelator Bicinchoninic Acid was used to corroborate the activation of CopB by Cu^{2+}. Copper reductases could be reducing the Cu from oxidation state (II) to (I), leading us to erroneous data. The ATPase activity of CopB-WT activated by Cu^{2+} or Cu^+ was measured in the presence of BCA (Fig 11). As expected, the Cu^+ chelator had a

negligible effect on the activity related to Cu^{2+} but annulled the activity of CopB in the Cu^{+}-containing assays. This experiment is demonstrating that there is no Cu^{+} activating CopB in the Cu^{2+}-containing assay.

A. fulgidus CopA ATPase activity was shown to be dependant on Cysteine concentration (Mandal *et al.*, 2002). In order to determine if CopB interacts with the metal ion in the same way, different concentrations of cysteine were added to the assay mixture. Fig 11 shows the ATPase activity of CopB in the presence of 20 mM cysteine. Cysteine had no effect on ATPase activation by Cu^{+}. However, it had a negative effect on Cu^{++} activation of the ATPase activity. It was proven that this enzyme does not need a metal-thiolate complex in order to interact with the metal ion.

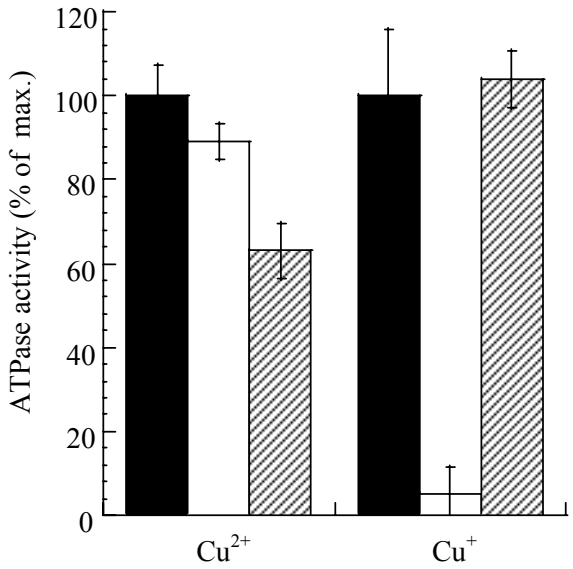

Fig 11: Effect of cysteine and BCA on the activation of CopB-WT by copper. $CuSO_4$ was added to a final concentration of 1μM in all cases and 2.5 mM DTT was used to reduce the Cu when needed. BCA (□) or Cysteine (▨) was added to a final concentration of 100 μM or 20mM, respectively. (■): Control.

Copper affinity curves were performed for CopB-WT and CopB-T with the purpose of determining apparent kinetic parameters. We wanted to establish whether the absence of the His-rich MBD had any effect on those parameters. Copper at both redox states (I)

and (II) independently activated CopB-WT and CopB-T with high apparent affinity (Fig. 12). The mutant protein showed a slightly higher affinity towards both copper redox states than the CopB-WT (Cu^{2+}: CopB-WT $K_{1/2} = 0.28 \pm 0.09$ μM; CopB-T $K_{1/2} = 0.12 \pm 0.03$ μM) and (Cu^{+}: CopB-WT $K_{1/2} = 0.38 \pm 0.2$ μM; CopB-T $K_{1/2} = 0.08 \pm 0.08$ μM).

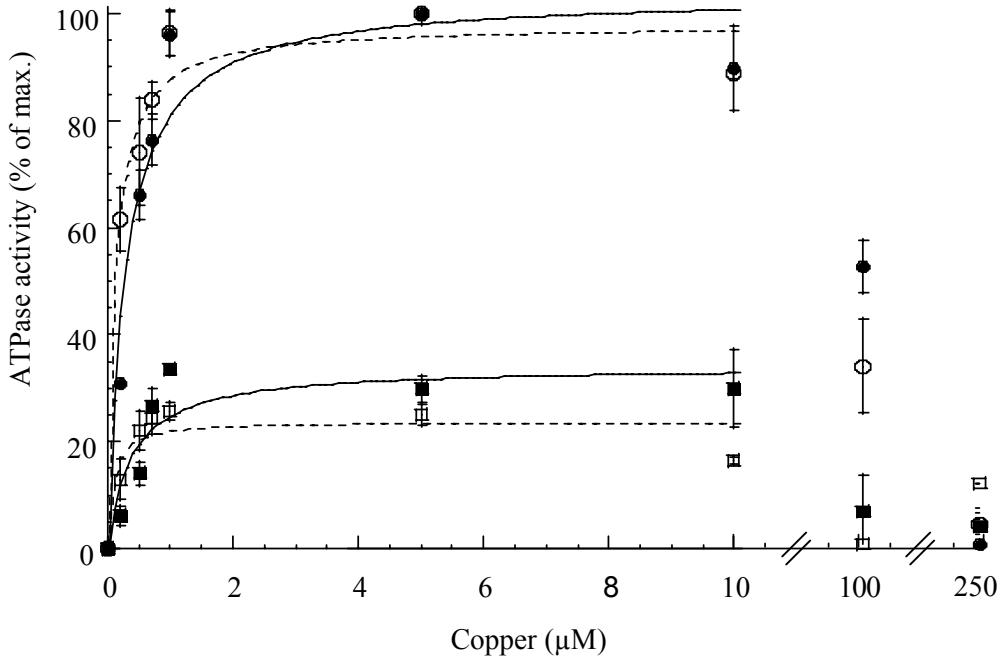

Fig 12: Dependence of CopB-WT and CopB-T ATPase activity on Cu^{2+} and Cu^{+}. The ATPase activity was measured in the presence of different concentrations of Cu^{2+} (●,CopB-WT; ○, CopB-T) and Cu^{+} (■,CopB-WT; □, CopB-T) ions. Data was fitted yielding the following parameters: CopB-WT: Cu^{2+} $K_{1/2} = 0.28 \pm 0.09$ μM; Cu^{+} $K_{1/2} = 0.38 \pm 0.2$ μM; CopB-T: Cu^{2+} $K_{1/2} = 0.12 \pm 0.03$ μM; Cu^{+} $K_{1/2} = 0.08 \pm 0.08$ μM. CopB-WT $V_{max} = 5.0 \pm 0.3$ μmol/mg/h and CopB-T $V_{max} = 3.0 \pm 0.3$ μmol/mg/h.

The V_{max} in the presence of Cu^{+} (CopB-WT = 34 ± 4%; CopB-T = 24 ± 3%) was between one fourth and one third of the value obtained for Cu^{2+}. CopB-WT and CopB-T exhibit similar levels of expression and V_{max} = 5.0 ± 0.3 μmol/mg/h and 3.0 ± 0.3 μmol/mg/h, respectively. The 40% decrease in the V_{max} yielded by CopB-T in comparison with the WT enzyme shows that the cytoplasmic N-terminus MBD is

necessary in order to have a fully active protein but is not essential for the enzyme to function. Copper at concentrations over 10 μM reduced the ATPase activity not only for CopB-WT but also for CopB-T. If the metal ion is present in the media at high concentrations, it can bind the phosphoenzyme intermediate (with very low affinity) after the releasing of the ion that is being transported. This interaction will lead in a reduction of the turnover of the enzyme. Finally, the shapes of the curves for either CopB-WT or CopB-T do not show any cooperativity. This finding is interesting because in addition to the MBD in H6 (CPH), CopB has the cytoplasmic MBD with 17 His that is not known how many binding sites presents.

3.6. ^{64}Cu uptake experiments

We already demonstrate that copper (II) was activating the enzyme. The next step was to prove that Cu^{2+} was also being transported. We used everted vesicles and the copper isotope ^{64}Cu to accomplish this goal. Bacteria carrying genes for CopB-WT or CopB-T were induced and harvested. In parallel, untransformed bacteria underwent the same process and everted membrane vesicles were prepared from all three conditions. Several temperatures were assayed and it was found that the optimum temperature for ^{64}Cu uptake was at 55º C (data not shown). The different temperature used for this assay (lower than with ATPase activity assays) is presumably due to a higher sensitivity of mesophilic vesicles to temperature. The different vesicles were incubated in the presence or absence of ATP and plotted as the difference between both conditions (Fig 13). CopB-WT initially presented a higher speed in the uptake of ^{64}Cu than the CopB-T.

Observations within the linear part of the curves yielded two fold higher uptake values for CopB-WT (200 ± 6 pmol/mg/min) over the CopB-T (80 ± 47 pmol/mg/min).

Control vesicles did not showed any ATP-related transport along the time-course experiment.

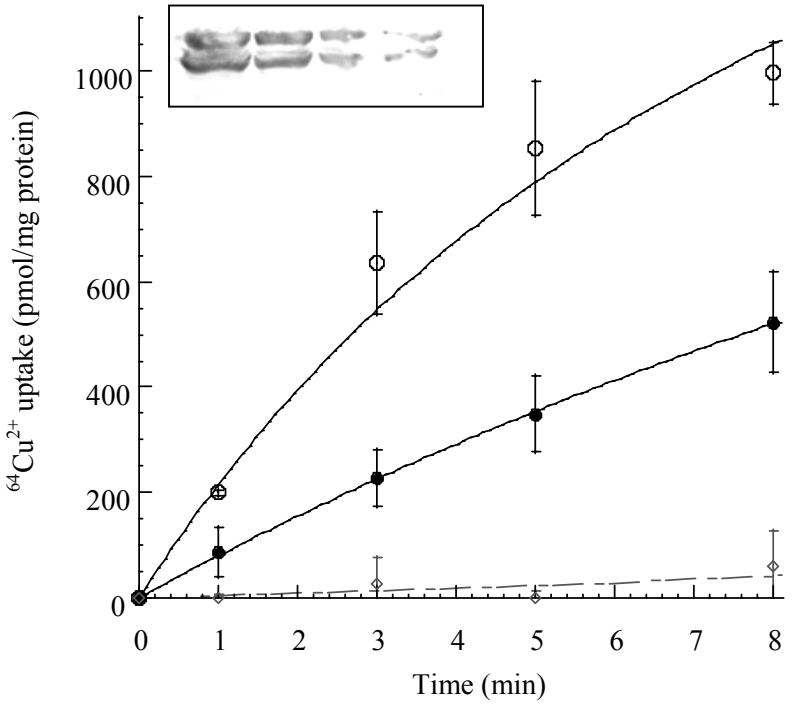

Fig 13: $^{64}Cu^{2+}$ uptake into everted vesicles. Everted vesicles were obtained from untransformed (◇), arabinose-induced pBAD/TOPO- CopB-WT (○) or pBAD/TOPO- CopB-T (●) *E. coli* as described in "Experimental Procedures". The experiment was performed at 55º C and $^{64}Cu^{2+}$ was added to a final concentration of 5 µM in all the cases. The tubes were preincubated for 1 min and the uptake reaction was started with the addition of 3 mM ATP. Aliquots of the different treatments were taken at the indicated times, filtered through 0.2 µm nitrocellulose filters and the radioactivity was measured with a scintillation counter. Results are shown as the difference between the values ± ATP. **Inset:** Immunoblot of serial dilution comparing the level of expression of CopB-WT (top) and CopB-T (bottom). Serials dilutions (1:2) were performed starting with 15 µg of each membrane loaded in the same lanes.

3.7 Interaction of CopB-WT and CopB-T 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 $E_2 \blacktriangleright E_1$ and as a consequence, the turnover. With the goal of obtaining this kinetic parameter and understand if the truncation of the protein has any affect, the dependence of ATPase on ATP was studied. Fig 14 shows the apparent affinity of CopB-WT and CopB-T towards ATP in the sub-mM range. CopB-WT (ATP $K_{1/2}$ of 0.11 ± 0.02 mM) and CopB-T (ATP $K_{1/2}$ of 0.06 ± 0.02 mM) exhibit similar apparent affinities towards ATP. This observation suggests that the lack of cytoplasmic MBD is not affecting the $E_2 \blacktriangleleft\blacktriangleright E_1$ equilibrium.

The high affinity interaction of P-type ATPases with ATP (μM range) leads to phosphorylation of the enzyme at the aspartic acid residue in the ATP binding site (Fig 2). The levels of phosphoenzyme of CopB-WT and CopB-T were measured to evaluate the effect of the truncation on phosphorylation. Phosphorylation experiments were performed as described in Mandal e*t al*., (2002). The temperature was lowered to 37° C to reduce turnover and 20% Me_2SO was added to the assay mix to stabilize the phosphorylated form of the enzyme. CopB-WT or CopB-T containing membranes were activated by 1 μM Cu^{2+} or Cu^{+} and phosphorylated by [γ-^{32}P] ATP. Results are shown in Fig 15. CopB-WT presented a maximum level of phosphorylation in the presence of Cu^{2+}. As expected, Cu^{+} also activated the enzyme leading to phosphorylation but to a lesser extent. The phosphorylation of the CopB-T did not show any difference compared with the CopB-WT. The lack of the cytoplasmic MBD did not affect the phosphorylation

step, suggesting no conformational change of the protein at the ATP binding site. Tubes with no metal added also presented some degree of phosphorylation, presumably due to metal contamination.

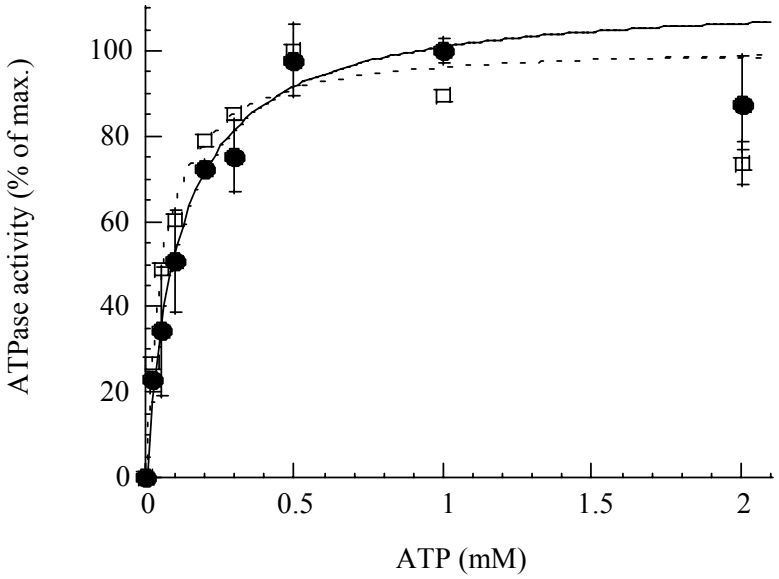

Fig 14: Dependence of CopB-WT and CopB-T ATPase activity on ATP. The Cu^{2+}-ATPase activity was determined as indicated under "Experimental Procedures" and ATP concentration varied as indicated. Data is shown as % of the highest ATPase value. The CopB-WT yielded an ATP $K_{1/2}$ = 0.11 ± 0.02 mM and the CopB-T = 0.06 ± 0.02 mM. $CuSO_4$ was added to a final concentration of 1 μM.

The effect of the truncation of the enzyme (lower V_{max}) could not be explained by modification of the affinity towards the metal ion (Fig 12) or changes in the $E_2 \leftrightarrow E_1$ equilibrium (Fig 14). We suspected that the lack of N-terminus MBD would be influencing the transition E_2P to E_2, shown to be the rate limiting step of the catalytic cycle (Mandal *et al.,* 2002). With this goal we studied the dephosphorylation rate of CopB-T and CopB-WT. Dephosphorylation experiments were carried out using Cu^{2+} activated CopB-WT or CopB-T-containing membranes and 1μM [γ-^{32}P] ATP. EDTA at a concentration of 5 mM was used to avoid "new" phosphorylation.

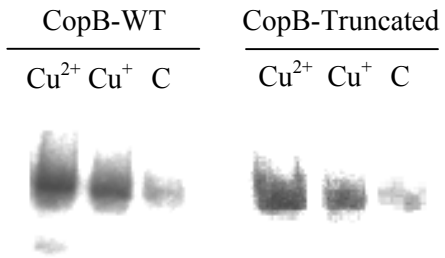

Fig 15: Phosphorylation of CopB-WT and CopB-T by [γ-^{32}P] ATP. Membranes were incubated at 37°C during 30s. CopB-WT and CopB-T were activated by Cu^{2+} or Cu^{+} and phosphorylated in the presence of 1μM ATP. Samples were centrifuged and loaded in acidic gels. Gels were dried and exposed to imaging plates. Pictures were taken using a phosphoimager.

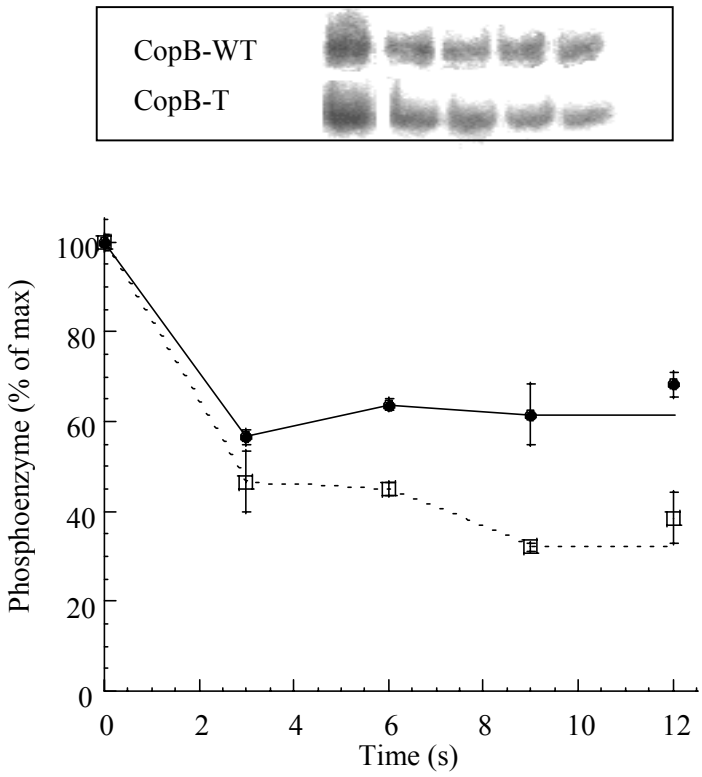

Fig 16. Dephosphorylation of CopB-WT and CopB-T. CopB-WT (□) and CopB-T (●) were phosphorylated in the presence of 1 μM Cu^{++} and 1μM [γ-^{32}P] ATP as described in Methods section. 5 mM EDTA was added at different times to phosphorylated membranes. The reaction was stopped with acid. Samples were loaded in acidic gels. The gels were dried and exposed in phosphoimager. No curve fitting was attempted.

Fig. 16 shows the levels of phosphoenzyme of CopB-WT and CopB-T at the specified times. CopB-WT and CopB-T showed a high level of dephosphorylation within the first 3 seconds. However, the CopB-WT showed a higher degree of dephosphorylation than the CopB-T (> 20%), suggesting a faster turnover. Truncation of CopB leaded in a more stable phosphoenzyme which would explain the reduced V_{max}.

4. DISCUSSION

CPx-ATPases are a relatively new subfamily of proteins that belong to the family of the P-type ATPases. More than 250 members of this subfamily have been found (http://biobase.dk/~axe/Patbase.html). CPx-ATPases can be subdivided in 6 groups named IB-1 to 6 based in ion selectivity determinants (Argüello, *unpublished*). The cytoplasmic MBD sequences can vary within CPx-ATPases, showing different motifs such as the His-rich or CxxC (Odermatt *et al.,* 1993; Axelsen and Palmgren, 1998). With the improvement of the genetic databases, His-rich CPx-ATPases have been found in different organisms (Lee *et al.*, 2001; Herrmann *et al.*, 1999; Axelsen and Palmgren, 1998). However, including the *A. fulgidus* CopB only 16 of these sequences contain a CPH in H6, though belonging to the IB-4 group. What is not known is if the presence of Histidines at both MBDs of IB4-ATPases confers some special characteristic to these transporters.

Nitrogens of the imidazolium rings of Histidines are hard Lewis bases and are the preferred ligand for Cu^{2+} (an intermediate Lewis acid). On the other hand, thiol groups of Cysteines are soft Lewis bases and prefer soft acids like Cu^{+} (Hughes, 1988). When the ligands are part of proteins, additional variables such as the stereochemistry of the protein molecule and the competition between the metal and H^{+} for the ligand donor site should be also considered (Hughes, 1981). Histidine has a $P_{KR} = 6$, which is very close to the pH used in this work. Moreover, the pH used in this research is very similar to the pH used in experiments conducted on the CopB ortholog in *E. hirae* (Solioz and Odermatt, 1995). It is well known for some N-terminus His-rich proteins, such as Cu^{2+}/Zn^{2+} SODs, that

particular His residues coordinate the metal ion and a mutation in these sites prevents this interaction (Batisttoni, 2001). CopB presents 17 His residues at the cytoplasmic N-terminus MBD and also a His is flanking the conserved Proline residue at the CPx motif. Taking into account the discussed above, IB-4 CPx-ATPases would be more likely to interact with Cu^{2+} than Cu^{+}.

The experimental evidence presented in this work demonstrates that CopB from *A. fulgidus* is a Cu^{2+} ATPase. It is a fundamental concept for P-type ATPases that the ATP hydrolysis is coupled to the translocation of the ion (Glynn, 1985), and as shown in Fig 10, the ATPase activity of CopB in presence of Cu^{2+} was approximately 40% higher than Ag^{+} and 70% more than Cu^{+}. Using the copper isotope ^{64}Cu we were able of demonstrate that Cu^{2+} was not only activating the enzyme, but also being transported (Fig 13). In agreement, CopB activated by Cu^{2+} reached higher levels of phosphorylation than under activation with Cu^{+} (Fig 15). As additional prove of the activation of this enzyme by Cu^{2+}, the ATPase activity of CopB was measured in the presence of BCA (Cu^{+} chelator). The experiment showed no variation in the ATPase levels, confirming our previous results.

The ortholog of *A. fulgidus* CopB in *E. hirae* shows a high homology of sequence (41.6%). *E. hirae* CopB was the first characterized IB-4 CPx-ATPase (Solioz and Odermatt, 1995). It was reported as a Cu^{+}/Ag^{+} ATPase in spite of the previously discussed structural characteristics. Through the different reports that show *E. hirae* CopB as a monovalent cation transporter, some insights that show the protein as a Cu (II) transporter can also be found. *E. hirae* ΔCopB cells were unable to grow in $CuSO_4$ supplemented media (Odermatt *et al.*, 1993) and they bioaccumulated five fold more

copper than the CopB-WT (Odermatt *et al.*, 1994) even in the presence of CopA, which ortholog was reported as Cu^{+} extrusion pump (Mandal *et al.*, 2002). Moreover, in the same bioaccumulation experiment ΔCopA cells carrying a functional CopB were shown to present normal cytoplasmic levels of copper. This indicates that the copper inside the cells was in the oxidation state (II), weakening the alternative hypothesis of reductases supposedly reducing the Cu (from II to I). In addition, ^{64}Cu uptake measurements into native vesicles (Solioz *et al.,*. 1995) had shown that *E. hirae* CopB transported more copper under non reducing conditions. The expression of *E. hirae* CopB was also shown to be activated by $CuSO_4$, $AgNo_3$ and $CdCl_2$ (Odermatt *et al.*, 1993). All this data suggests that IB-4 ATPases are $Cu^{2+}/Cu^{+}/Ag^{+}$ transporters.

Our group has already reported the characterization of *A. fulgidus* CopA as a Cu^{+}/Ag^{+} ATPase (Mandal *et al.*, 2002). CopA was active at 75° C, 400 mM NaCl and pH 6.1, conditions that are very similar to the used in this work for CopB. The enzyme also showed interaction with ATP with two apparent affinities, showing an ATPase $K_{1/2}$ in the same order of magnitude of the one for CopB. The ATPase activity of CopA was stimulated by Cu^{+} with a $K_{1/2} = 2.1 \pm 0.3$ μM, showing less affinity for copper than CopB (Cu^{2+} $K_{1/2} = 0.28 \pm 0.09$ μM; Cu^{+} $K_{1/2} = 0.38 \pm 0.2$ μM). Under physiological conditions these enzymes probably interacts with different metal complexes and metal chaperones which may vary their affinities towards the metal ions. It was shown that CopA needed the addition of Cysteine in the assay mixture in order to be activated by Cu^{+}. This dependence on metal-thiolate complexes was not seen for CopB (Fig 11). Finally, CopA showed no ATPase activity upon activation of by Cu^{2+}. In this way can be understand

how the different pools of copper (oxidation state I and II) are extruded from the cell, conferring to *A. fulgidus* resistance to a polluted environment.

In order to understand the functional role of the cytoplasmic MBD, CopB lacking the N-terminus domain (CopB-T) was also assayed. CopB-T showed activation by the same metals than CopB-WT and to the same relative extent. It is significant that CopB-T preserves the same metal selectivity. It shows that the cytoplasmic MBD is not an ion selectivity determinant. CopB-T showed a 40 % decrease on the ATPase activity compared with CopB-WT (Fig 12). This data is consistent with the results obtained in the vesicle uptake experiment (Fig 13). The lack of MBD did not affect the affinities for Cu^{++} and $Cu^{+,}$ as shown in the copper affinity curves (Fig 12). The phosphorylation level of the truncated enzyme was also determined under activation by Cu^{++} and $Cu^{+.}$ CopB-T presented the same level of phosphoenzyme than CopB-WT, showing that the equilibrium between E_1 and the phosphoenzyme was not affected. On the other hand, CopB-T showed a reduced rate of dephosphorylation (Fig 16), demonstrating that the lack of cytoplasmic MBD modify the equilibrium $E_2P \leftrightarrow E_2$. It was proved for *A. fulgidus* CopA that the rate limiting step in the reaction is the dephosphorylation (Mandal *et al.,* 2002), thus any modification in this step would lead in changes in the V_{max}. In this way can be suggested a regulatory role for the cytoplasmic MBD, where binding of the metal ion leads into modification of the dephosphorylation rate. This putative regulatory role is in agreement with the mechanism proposed for the IB-1 CPx-ATPase WDP (Tsivkovskii *et al.,* 2001). In this report they showed a copper dependant interaction between the purified ATP binding domain and the N-terminus MBD, where the interaction diminish towards metal ion binding. They also have shown that the interaction induces a

conformation change of the ATP binding domain. In summary, “CxxC” and “His-rich” ATPases may have similar regulatory mechanisms of enzyme turnover, where metal binding to N-MBD controls the rate limiting step of the catalytic cycle.

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