The ligand dependent interactions between cytoplasmic domains in Cu^{+} transporter, *Archaeoglobus fulgidus* CopA

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Abstract

Cu^+-ATPases receive Cu^+ from specific chaperones via ligand exchange and subsequently drive the metal efflux from the cell cytoplasms. Cu^+-ATPases have two transmembrane metal binding/transport sites (TM-MBS) and various cytoplasmic domains: the actuator (A-domain) and ATP binding domains (ATPBD), and regulatory N-terminal metal binding domains (N-MBD). *Archaeoglobus fulgidus* CopA, the Cu^+-ATPase used in these studies, contains a single N-MBD and an apparently non-functional C-terminal MBD. The Cu^+ dependent interaction of N-MBD and ATPBD was postulated as a possible mechanism for enzyme regulation. The Cu^+ transfer from the chaperone to CopA is independent of the N-MBD capability to bind Cu^+. Therefore, we hypothesized that ligand (Cu^+ or nucleotide) binding to cytoplasmic domains might affect the interactions between the cytoplasmic domains. To test these ideas, the interactions among isolated cytoplasmic domains were characterized. Studies using isolated domains showed that while the N-MBD interacts with ATPBD, the presence of Cu^+ or nucleotide (ADP) prevents this interaction. The N-MBD does not interact with the A domain. Alternatively, the C-MBD interacts with both ATPBD and A-domains in a ligand independent fashion. Only one Cu^+ is transferred to CopA in absence of nucleotides, while the presence of ADP allows full loading of TM-MBS. These results suggest that the ligand binding affects the interactions between the cytoplasmic domains, and also change the conformation of CopA to help it accept the second Cu^+ from chaperone.

Key Words: P_{IB}-type ATPase, CopA, cytoplasmic domain, copper.

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Table of contents

ABSTRACT i

ACKNOWLEDGEMENTS ii

TABLE OF CONTENTS iii

LIST OF FIGURES v

LIST OF ABREVIATIONS vi

1. INTRODUCTION **1**

1.1. Metals and life *1*

1.2. P-type ATPases *4*

1.3. P_{1B}-type ATPases *6*

1.4. P_{1B}-type ATPases in Archaeoglobus fulgidus *7*

1.5. Catalytic Mechanism of P_{1B}-type ATPases *9*

1.6. Cytoplasmic domains in P_{1B}-type ATPase *11*

2. Materials and methods **16**

2.1. Constructs for isolated cytoplasmic domains expression *16*

2.2. CopA cytoplasmic domains preparation *16*

2.3 Domain-domain co-purification assays using Ni^{2+} resin *17*

2.4. Co-purification of interacting domains using StrepTactin resin *18*

2.5. Co-purification of Na^+/K^+-ATPase ATPBD with CopA N-MBD *18*

2.6. Cross-linking of interacting cytoplasmic domains *19*

3. Result **20**

3.1. The ligand dependent interactions between N-MBD and ATP-BD *20*

3.2. The N-MBD does not interact with A-domain *24*

3.3. The ligand independent interaction between C-MBD and ATP-BD *25*

3.4. The Cu^{+} independent interaction between C-MBD and A-domain *28*

4. Discussion **30**

4.1. Regulatory interaction between N-MBD and ATP-BD *30*

4.2. The interaction with the non-functional C-MBD *31*

4.3. Domain-domain interaction and catalytic cycle. *32*

5. References 34

LIST OF FIGURES AND TABLE

Figure 1. Copper homeostasis mechanisms in *E. coli*. 3

Figure 2. Phylogenic tree of P-type ATPases. 5

Figure 3. Schematic Representation of the Membrane Topology of P_{1B}-type ATPases. 9

Figure 4. Scheme of the catalytic cycle of P-type ATPases. 11

Figure 5. SR Ca^{2+}-ATPase structures and models. 14

Figure 6. The Structure of CopA. 15

Figure 7. Cu^{+} dependent interaction between N-MBD and ATP-BD. 21

Figure 8. Cu^{+} dependent interaction assay between Strep-tagged N-MBD and His-tagged ATP-BD. 22

Figure 9. Cross-linking assay between Strep-tagged N-MBD and His-tagged ATP-BD. 22

Figure 10. Nucleotide dependent interaction between N-MBD and ATP-BD. 23

Figure 11. Interaction assay between N-MBD and Na^{+}/K^{+}-ATPase ATP-BD. 24

Figure 12. Absence of interaction between N-MBD and A-domain. 25

Figure 13. Cu^{+} independent interaction between C-MBD and ATP-BD. 26

Figure 14. Nucleotide independent interaction between C-MBD and ATP-BD. 27

Figure 15. Interaction between C-MBD and A-domain. 28

Figure 16. Interaction assay between Strep-tagged C-MBD and BSA. 29

Figure 17. Proposed catalytic and transport cycle of Cu^{+}-ATPases. 32

TABLE 1. CopA cytoplasmic domain constructs used in this study 16

LIST OF ABREVIATIONS

A-domain: Actuator domain

AfCopA: *Archaeoglobus fulgidus* CopA

APS: Ammonium persulfate

ATP-BD: ATP binding domain

BCS: bathocuproindesidulfonic acid
BP fractions: bound protein fractions

C-MBD: C-terminus metal binding domain

CCS: copper chaperone for Cu,Zn-SOD

DTT: Dithiothreitol

IPTG: isopropyl β-D-thiogalactopyranoside

MNK: Menkes disease protein

N-domain: nucleotide binding domain

N-MBD: N-terminal metal binding domain

P-domain: phosphorylation domain

SOD: Super oxide dismutase

SR: Sarcoplasmic reticulum

SERCA: Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase

TM: Transmembrane segment

TM-MBSs: transmembrane metal binding sites

WNDP: Wilson's disease protein

XIAP: X-linked Inhibitor of Apoptosis Protein

UP factions: unbound protein fractions

1. Introduction

1.1 Metals and life

It has been estimated that a quarter of the known enzymes need a specific metal ion for their proper functions. These enzymes can be divided into two kinds: metalloenzymes and metal activated enzymes. For the metal activated enzymes, the metal is loosely bound to the enzymes, and the purified enzyme may need to be activated by the addition of metal ions. However, the metal is much more strongly bound to metalleoenzymes and retained even after the purification. In metalloproteins, metals could have several structural or catalytic roles such as: 1) constituting enzyme active sites; 2) stabilizing enzyme geometry structure; 3) forming weak-bonds with substrates contributing to their orientation to support chemical reactions; and 4) stabilizing charged transition states (Fraga 2005).

Heavy metal ions, including iron (Fe), copper (Cu), and zinc (Zn) and manganese (Mn), could play many essential roles in cells. Due to having unpaired electrons, Cu, Fe and Mn participate in redox reactions in enzyme active sites (Fraga 2005). They act as cofactors in enzyme reactions including hydrolysis, redox and group transferring (Pena et al 1999).

Copper is an essential trace element with key physiological and biochemical functions. Copper is a redox-active metal that is used by almost all organisms and could fluctuate between the oxidized (Cu^{2+}) and reduced (Cu^{+}) states. With this change in redox state, copper could coordinate to different ligands such as carboxylate oxygen, imidazole nitrogen, thiolate and thioether sulphur groups. Cu-containing proteins are involved in numerous biological processes in the cell, including oxidative phosphorylation

(cytochrome C oxidase), antioxidant activity (superoxide dismutase (SOD)), iron metabolism (ceruloplasmin), connective tissue formation (lysyl oxidase), innate immune system (tytosinase/laccase), and apoptosis (X-linked Inhibitor of Apoptosis Protein (XIAP)) (Linder & Goode 1991, Pena et al 1999, Turski & Thiele 2009, Vulpe et al 1993). However, the excess amount of free Cu is extremely toxic. The transition between a stably oxidized-Cu^{2+} and unstable reduced-Cu^{+} can generate free radicals such as superoxide radical and hydroxyl radical. Those highly reactive and oxidant molecules can react with many biomolecules such as nucleic acids or membrane lipids and may eventually lead to cell death.

Thus, cell has to have highly regulated mechanisms for Cu homeostasis (Figure 1). In eukaryotes, Cu is taken into the cell by specific high affinity uptake systems such as CTR1 in the CTR family (De Feo et al 2009). In bacteria, Cu enters the periplasm by an unknown mechanism, possibly through the porins, OmpC and OmpF (Li et al 1997).

After entering into the cell, Cu is bound to the Cu chaperones. The Cu chaperones are small cytosolic proteins which could carry out Cu delivery to membrane-bound proteins, such as Cu-transporting ATPases, and to Cu-dependent enzymes. The Cu chaperones are also able to bind and deliver Cu ions to intracellular compartments and insert Cu into the active sites of specific partners, Cu-dependent enzymes. Cu chaperones include Atox1 (Atx1 in yeast), CopZ and CCS (copper chaperone for Cu, Zn-SOD). Because of the existence of Cu chaperones, there are almost no free cytosolic Cu ions (Field et al 2002, Vulpe & Packman 1995).

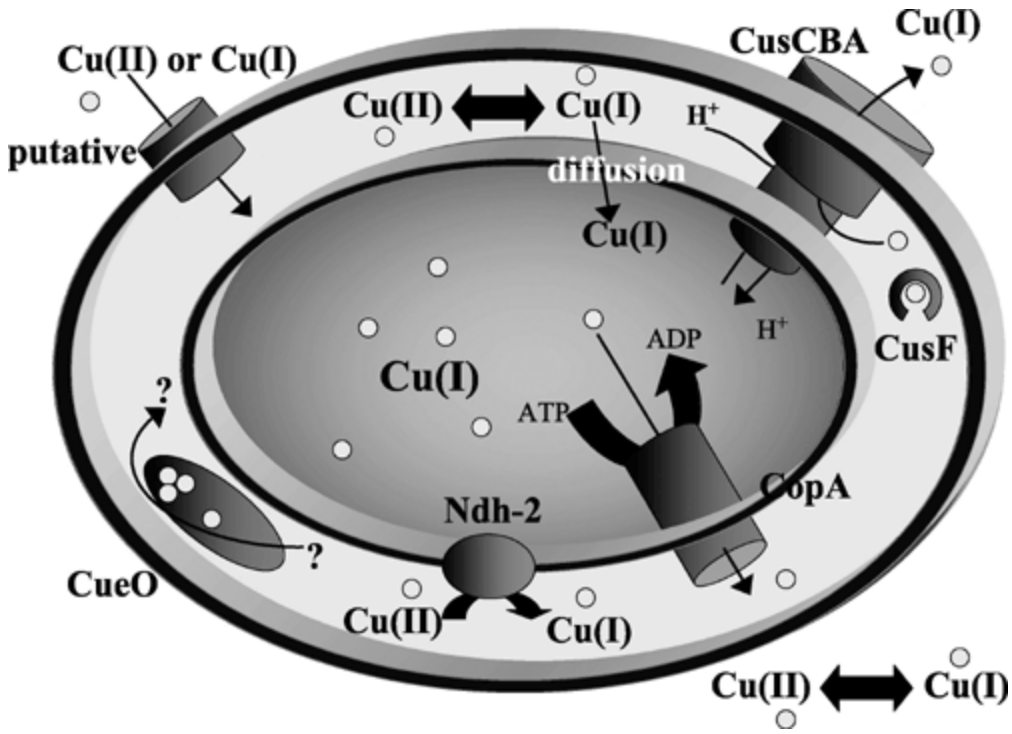

Figure 1. Copper homeostasis mechanisms in *E. coli.* Shown are the most relevant homeostatic systems. CopA is a Cu^{+}-translocating P-type ATPase, CusCFBA a four-component copper efflux pump. Cu enters the periplasm by an unknown mechanism. (Rensing & Grass 2003).

When the Cu concentration in the cell is high, Cu needs to be exported out of the cell by Cu transporters. The *E. coli* CusCFBA system is exclusively found in Gram-negative bacteria that could pump Cu^{+} and Ag^{+} out to the extracellular space using the proton gradient across the inner membrane as an energy source (Rensing & Grass 2003). There are four proteins in this system, which are CusA, CusB, CusC and CusF. These proteins are all the members of the RND1 (resistance, nodulation, division) or CBA type of efflux systems (Tseng et al 1999). It is also well known that Cu is exported out from the cells via a type of transporters named Cu specific P_{IB}-type ATPases, which belongs to the P-type ATPases. We will have a more detailed discussion about the P-type ATPases in the next sections.

In fact, because of the homeostatic mechanisms together with efficient chelating agents and chaperones, the concentration of free Cu ions in the cell is below 0.2 pM, which means almost only one free Cu per cell (O'Halloran & Culotta 2000).

1.2 P-type ATPase

P-type ATPases are a family of transmembrane proteins 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 (Axelsen & Palmgren 1998, Kühlbrandt 2004, Lutsenko & Kaplan 1995). P-type ATPases appear early in evolution and are key proteins in the maintenance of metal homeostasis in all organisms (Axelsen & Palmgren 2001, Axelsen & Palmgren 1998, Mills et al 2005, Mills et al 2003, Williams & Mills 2005). The main structural characteristics of these enzymes are six to ten transmembrane segments (TM1-TM10) and one ATP binding domain (ATP-BD). The highly conserved DKTGT domain, located in the ATP-BD, is the landmark of P-type ATPase. It is well known that the phosphorylation of the conserved Asp residue in this sequence drives the key conformational change in the protein(Axelsen & Palmgren 1998, Kühlbrandt 2004, Lutsenko & Kaplan 1995).

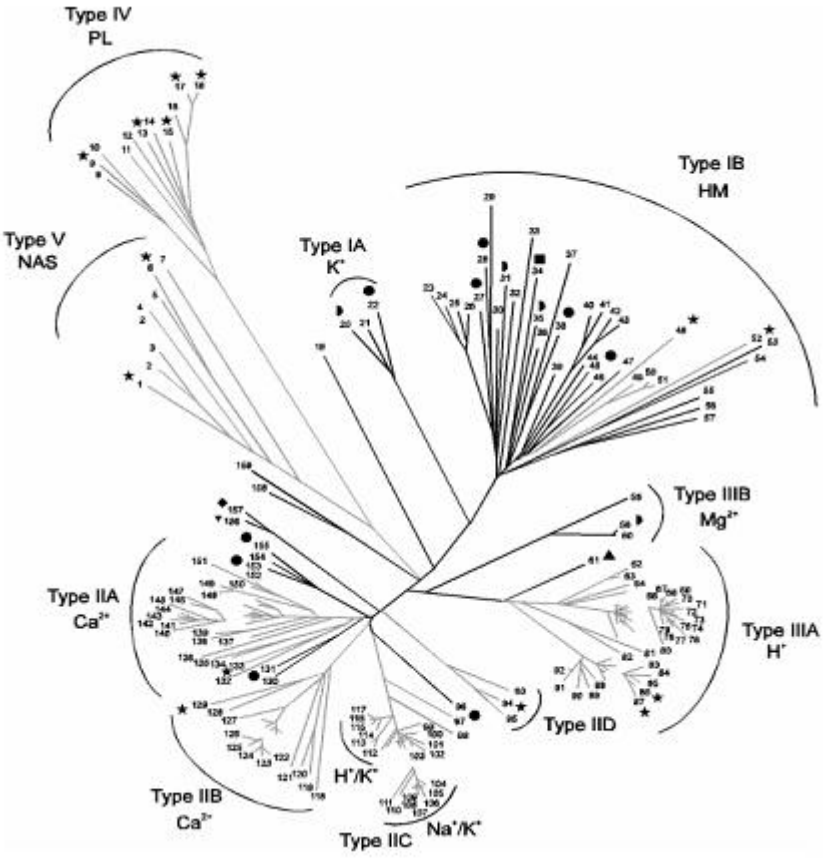

Figure 2. Phylogenic tree of P-type ATPases (Axelsen & Palmgren 1998).

P -type ATPases are divided into five subgroups based on the protein aliment and putative ion selectivity (Fig. 2). The P_I group is divided into: P_{IA}: bacterial Kdp-like ATPases and P_{IB}: heavy metals 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} are: H^+ and Mg^{2+} transporters. Group P_{IV} are lipid transporters. P_V is a group with unknown substrate specificity (Argüello 2003, Axelsen & Palmgren 1998).

1.3 P_{1B}-type ATPases

P_{1B}-type ATPases have been found in many organisms including: archaea (Mandal et al 2002), bacteria (Okkeri & Haltia 1999, Rensing et al 2000, Rogers et al 1991), plants (Eren & Argüello 2004, Gravot et al 2004, Tabata et al 1997, Thomine et al 2000), yeast (Bull & Cox 1994, Catty et al 1997), and animals(Bull et al 1993, Lutsenko & Petris 2003, Vulpe et al 1993).

The sequence analysis suggests that most of P_{1B}-type ATPase contain 8 transmembrane fragments (TM) (Argüello 2003, Axelsen & Palmgren 2001, Axelsen & Palmgren 1998, Bull & Cox 1994, Lutsenko & Kaplan 1995, Solioz & Vulpe 1996). This has been experimentally confirmed in two bacterial enzymes, *Helicobacter pylori* CadA and *Staphylococcus aureus* CadA (Melchers et al 1996, Tsai et al 2002). However, we still could not eliminate the possibility that a small subgroup of P_{1B}-type ATPases only has 6 TMs (Argüello 2003).

The key feature of these proteins is that they all have a putative metal binding sequence "CPx" or "xPC" such as CPC, CPH, or SPC, in the 6^{th} TM just before the ATP-BD (Argüello 2003, Argüello et al 2007, Solioz & Vulpe 1996). This sequence is essential for the enzyme activity, because the mutations on this sequence can cause the enzyme work improperly (Mandal & Argüello 2003).

Another feature of P_{1B}-type ATPases is the presence of one to six N-terminus metal binding domain (N-MBD). Some P_{1B}-type ATPases also have C-terminus metal binding domain (C-MBD) (Eren & Argüello 2004, Eren et al 2006, Mandal & Argüello 2003). The cysteines from the conserved CxxC sequence in N-MBD have the ability to bind

both monovalent and divalent cations (Cu^{+}, Cu^{2+}, Zn^{2+} and Cd^{2+}) (Banci et al 2002, DiDonato et al 1997, Gitschier et al 1998, Jensen et al 1999, Lutsenko et al 1997). Instead of CxxC sequence, some ATPases have a His-rich N-MBD, for instance CopB (Argüello et al 2003, Mana-Capelli et al 2003).

The metal selectivity of P_{1B}-type ATPases is determined by the CPC sequence in TM6 plus signature sequences in TM7 and TM8. P_{1B}-type ATPases can be divided according by their ion selectivity into: P_{1B-1}: Cu^{+}, P_{1B-2}: Zn^{2+}, P_{1B-3}: Cu^{2+}, P_{1B-4}: Co^{2+}.

1.4 P_{1B}-type ATPases in *Archaeoglobus fulgidus*

Archaeoglobus fulgidus is a hyperthermophilic archeae. The sequence of its genome is available (Klenk et al 1997). The advantage to study enzymes from thermophilic organisms is that they are usually more stable and easy to purify (Mandal et al 2002).

The *A. fulgidus* has two P_{1B}-type ATPases, CopA and CopB. CopB has a CPH sequence in H6 and a His-rich N-MBD. Previous work from our laboratory has shown that CopB is a Cu^{2+}-ATPase and the His-rich N-MBD is not involved in the metal selectivity (Mana-Capelli et al 2003). CopA is a P_{1B1}-type ATPase which transports Cu^{+} or Ag^{+} out of the cell (Mandal et al 2002). CopA has the CPC sequence in TM6. It also contains an N-MBD and a C-MBD which both have the CxxC sequence for binding Cu^{+} (Fig. 3).

In out laboratory, we have already cloned and expressed the AfCopA in *E.Coli* as a fusion protein with a hexahistidine tag. The ATPase activity of CopA is 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^+ with lower affinity (V_{max} = 14.82 μmol/mg/h and a $K_{1/2}$ = 29.4 μM). Divalent Cu^{2+} or Zn^{2+} could not activate the enzyme. 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 (Ag^+, 1.40 nmol/mg; Cu^+, 1.08 nmol/mg) (Mandal et al 2002).

A. fulgidus CopA has two transmembrane metal binding sites (TM-MBSs). Both sites can be independently loaded with free Cu^+. However, their simultaneous occupation is associated with enzyme turnover. Site I is constituted by two Cys in transmembrane fragment 6 and a Tyr in TM7. An Asn in TM7 and Met and Ser in TM8 form Site II. Single site X-ray spectroscopic analysis indicates a trigonal coordination in both sites. This architecture is distinct from that observed in Cu^+-trafficking chaperones and classical Cu-proteins. Both sides have very high affinity of these sites for Cu^+ (Site I K_a=1.3 fM^{-1}, Site II K_a=1.1 fM^{-1} (González-Guerrero et al 2008).

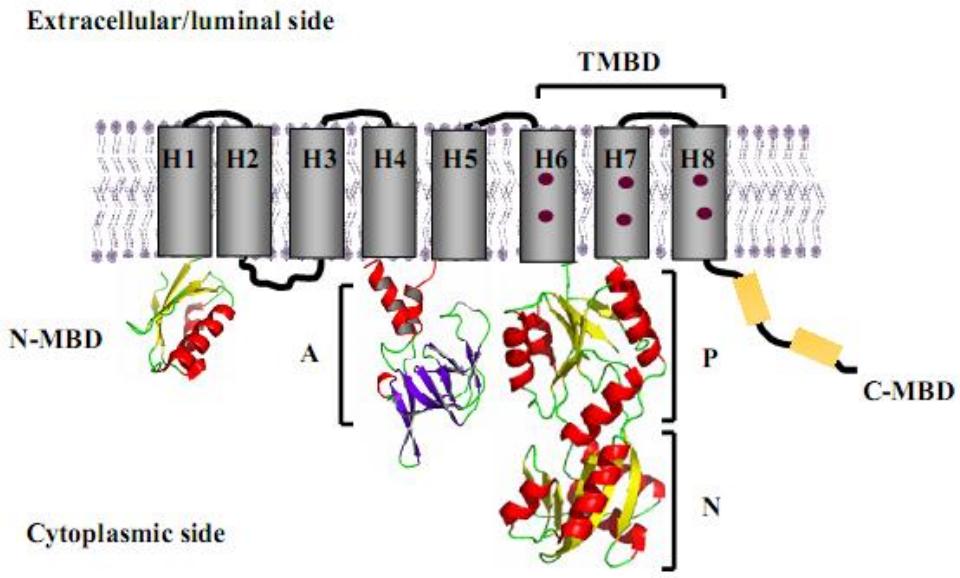

Figure 3. Schematic Representation of the Membrane Topology of P_{1B}-type ATPases. Transmembrane helices, H1-H8, are indicated. The relative locations and structure of Archaeoglobus fulgidus CopA actuator (A) domain and phosphorylation (P) and nucleotide (N) domains (Sazinsky et al 2006a, Sazinsky et al 2006b) are shown. To represent one of the repeats present in the N-terminus the human Menkes disease protein (MNK) fifth N-terminal metal binding domain (N-MBD) (Banci et al 2005) is depicted. The conserved amino acids in H6, H7 and H8 forming the transmembrane metal binding sites (TM-MBSs) are symbolized by red dots. The C-terminal metal binding domains (C-MBDs) with likely diverse structures are represented by yellow rectangles.

1.5 **Catalytic Mechanism of P_{1B}-type ATPases**

Like all other P-type ATPases, P_{1B}-ATPases transport metals across membranes following the classical E1/E2 Albers-Post catalytic cycle by changing the affinities of intercellular metal binding sites from high in E1 to low in E2 (Post et al. 1972) (Fig. 4). This transport mechanism has been well studied in P_2-ATPases such as Na^+/K^+-, Ca^{2+}-, and H^+/K^+-ATPases (Kaplan 2002, MacLennan et al 1997). In E1 state, the enzyme is first phosphorylated upon ATP binding with high affinity to the ATP-BD and metal binding to the TM-MBS from the cytoplasmic side. Upon phosphorylation, the metal is likely occluded within the TM region and thus is not accessible from either of the

membrane side. The subsequent conformational change allows for metal deoclussion, releasing to the extracellular (vesicular/luminal) compartment, and subsequent enzyme dephosphorylation. The enzyme then returns to the E1 conformation upon ATP binding to the E2 form with low affinity.

ATPase activity, metal transport studies, and individual step of the catalytic mechanism such as phosphorylation, dephosphorylation have been studied in eukaryote, prokaryote and archeal P_{1B}-type ATPases. These studies have been carried out with isolated enzyme or membrane preparation of Cu^+ or Zn^{2+} ATPases. (Eren & Argüello 2004, Fan & Rosen 2002, Mana-Capelli et al 2003, Mandal et al 2002, Sharma et al 2000, Tsivkovskii et al 2002, Voskoboinik et al 1998).

The noticeable difference between Cu^+-ATPases and other P-type ATPases such as Na^+/K^+ ATPase is that since there is no free Cu^+ in cell, the Cu^+ chaperone are responsible for delivering Cu to the enzyme. Our laboratory has already shown that the CopZ, the Cu^+ chaperone in *A. fulgidus*, could directly transfer Cu^+ to the transmembrane TM-MBSs of CopA (González-Guerrero & Argüello 2008). In non turn-over condition, CopZ only could deliver one Cu^+. However chaperone mediated Cu^+ transfer to the second TM-MBS occurs when ATP is bound to CopA. The observation that CopZ transfers Cu^+ indistinctly to either TM-MBS suggests that ATP bound to CopA is not making the second TM-MBS available but changing the conformation of CopA which helping the enzyme to accept the second Cu^+ from CopZ (González-Guerrero et al 2009). We hypothesize that movements of the cytoplasmic domains are associated with this conformational change.

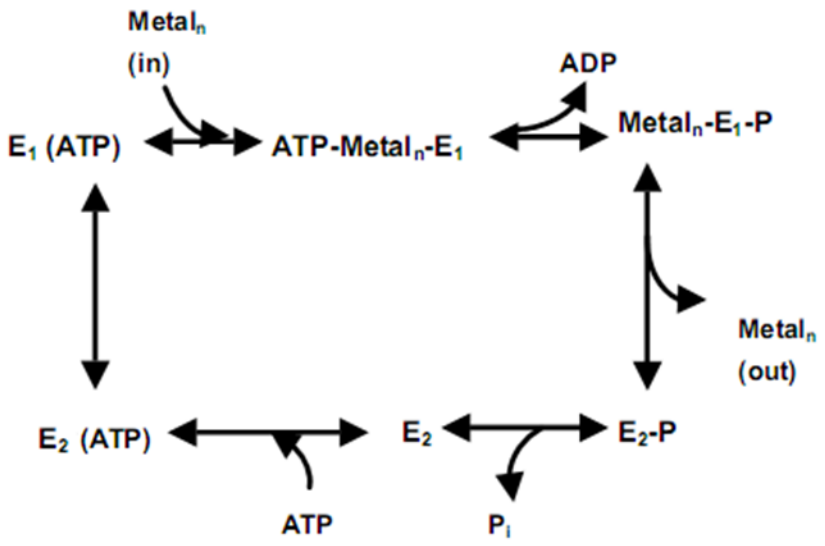

Figure 4. Scheme of the catalytic cycle of P-type ATPases. E1 and E2 are conformations of the ion pump with ion-binding sites facing the cytoplasm and extracellular medium, respectively. The metal ion is translocated using the energy of generated by ATP hydrolysis, which triggers conformational changes of the enzyme. Enzyme phosphorylation and dephosphorylation occur on the cytoplasmic side of the protein.

1.6 Cytoplasmic domains in P_{1B}-type ATPase

P_{1B}-type ATPases contain the ATP Binding domain (ATP-BD), the actuator domain (A-domain) and the metal binding domains (MBDs).The N-MBDs in P_{1B}-ATPases are 60–70 amino acids domains with a highly conserved CXXC metal-binding sequence (Arnesano et al 2002, Lutsenko et al 2003, Rensing et al 1999). The number of N-MBDs varies from one in bacteria and archaea to six in higher eukaryote ATPases. Cytoplasmic MBDs have a conserved βαββαβ fold and an invariant CXXC metal-binding sequence similar to the well-described Cu^+-chaperones, Atox1, Atx1, and CopZ (Arnesano et al 2002, Banci et al 2001, Wernimont et al 2000). N-MBDs have a regulatory role in controlling the enzyme turnover rate (Fan & Rosen 2002, Mandal & Argüello 2003, Voskoboinik et

al 1999). In addition, they are involved in the important sorting of Cu^+-ATPases observed in eukaryotes (Lutsenko et al 2007). Lutsenko and collaborators have shown that a protein construct containing the six N-MBDs present in the human ATP7B Cu^+-ATPase, interacts with the ATP-BD in a Cu^+ dependent manner (Tsivkovskii et al 2001). Recently obtained structures of *A. fulgidus* CopA show the physical proximity between its single N-MBD, the A-domain, and the ATP-BD (Wu et al 2008). These two observations would provide a structural basis for the regulatory role of cytoplasmic MBDs via Cu^+-dependent domain-domain interactions (Fan & Rosen 2002, González-Guerrero & Argüello 2008, Mandal & Argüello 2003, Tsivkovskii et al 2001, Voskoboinik et al 1999). However C-MBD does not appear to have a function role.

In 2006, the crystal structures of the isolated N-domain of human Cu^+-ATPase Wilson's disease protein (WNDP) and A-domain and ATP-BD of (AfCopA) were solved which shows the similar structures to those in P_2-type ATPases. This suggests that the same basic folding structure is responsible for their functional properties (Dmitriev et al 2006, Sazinsky et al 2006a, Sazinsky et al 2006b).

The A-domain is formed by the loop between TM4 and TM5 in P_{1B}-type ATPase. The A-domain shows a 10 β-strand core with 2 α-helices connecting the TMs and its folding shows significant similarity to the A-domain from a P_2-type Ca^{2+}-ATPase, SERCA1, while their sequence homology is only 26% (Toyoshima & Inesi 2004, Toyoshima & Mizutani 2004, Toyoshima et al 2004). In both, the conserved (S/T)GE(P/S) sequence appears to be located at the tip of a solvent accessible loop on the outer side of the A-domain. Like in SERCA1, it has been shown that this loop interacts with the ATP-BD during phosphorylation/dephosphorylation driving the rotation of the A-domain with

subsequent rearrangement of TMs (Olesen et al 2004, Toyoshima & Inesi 2004, Toyoshima & Mizutani 2004, Toyoshima et al 2004). This arrangement will cause metal be released from TM-MBS. Structural similarity of A-domain between the P_{1B}-type ATPases and that of SERCA1 might point to a similar metal releasing mechanism.

The ATP-BD, the large cytoplasmic loop between TM6 and TM7 of P_{1B}-ATPases containing the DKTGT sequence, is encompassed the nucleotide binding (N) and the phosphorylation (P) domains. The ATP-BD domain structure shows that the P- and N-domains are joined by two short loops which are called the hinged region (Sazinsky et al 2006b). The P-domain consists of a 6 stranded parallel β-sheet sandwiched between 3 short α-helices. This domain contains the conserved DKTGT sequence and shows similar folding to the P-domain of SERCA1 (Toyoshima et al 2000). The N-domain of AfCopA consists of six antiparallel β-sheet flanked by four short α-helices. Its structure is also very similar to N-domain in the WNDP, SERCA and KdpB independent by their homology.

The similar structures of the A, P, and N domain indicate that, in spite of the differences among the sequences of the different P-type ATPase, they share the similar structure, not only for binding ligands, but also for the critical conformational changes. The whole crystal structure of SERCA has been archived with different ligands in several conformations at high resolution level. Detailed comparison of the structures in the Ca^{2+} bound and unbound forms reveals that very large movements of the cytoplasmic domains and that they are mechanically linked with equally large rearrangements of transmembrane helices take place accompanying Ca^{2+} dissociation-binding. The most surprising discovery was that 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. 5). These arrangements of ATP-BD and A-domain are thought to be happened in P_{1B}-type ATPase too, and will affect the interactions between the cytoplasmic domains.

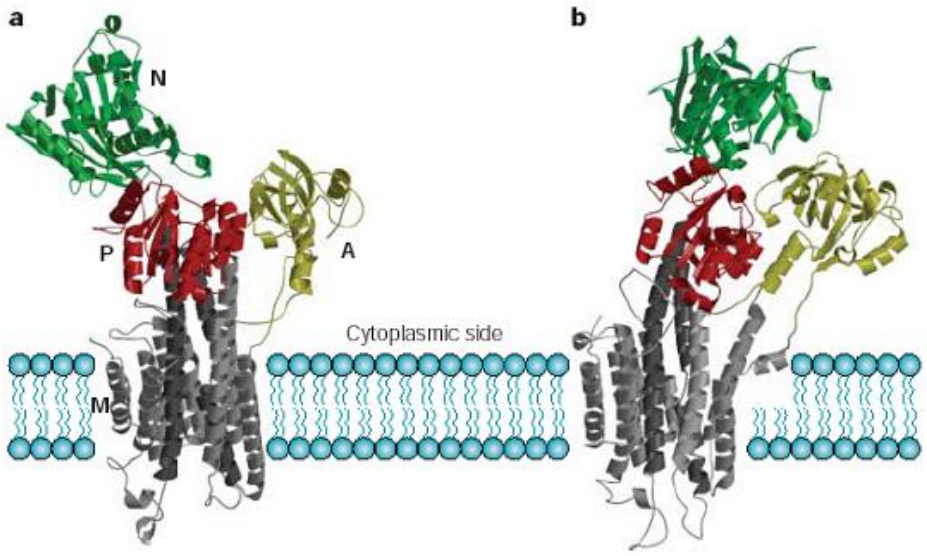

Figure 5. SR Ca^{2+}-ATPase structures and models. A. Ca^{2+}-ATPase in the E1 state ((Toyoshima et al 2000). B. SR Ca^{2+}-ATPase in the thapsigargin-inhibited E2 state (Toyoshima & Nomura 2002). The phosphorylation (P)-domain is shown in red, the nucleotide binding (N)-domain in green, the actuator (A)-domain in yellow, the membrane (M)-domain in grey. (Toyoshima & Nomura 2002).

A low resolution of whole CopA structure was obtained by cryoelectron microscopy. The structure shows that in the absence of ligands, the N-MBD is very close to both ATP-BD and A-domain (Wu et al 2008) (Fig. 6).

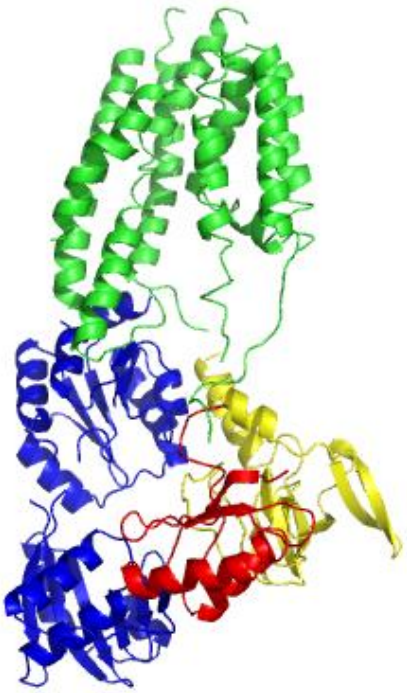

Figure 6. The structure of CopA. The N-MBD is shown in red, the ATP-BD in blue, the A-domain in yellow, the transmembrane fragment in green .

The ligands bound to CopA will cause the conformational change. Will this conformational change consequently affect other aspects of the enzyme properties? We suggest that it will cause the movement of the cytoplasmic domains and thus affect the interactions between these domains. To test this idea, we are using the individual isolated cytoplasmic domains to test the hypothesis that the interactions takes place between the cytoplasmic domains and ligand binding affects these interactions.

2. Materials and methods

2.1 The construct of isolated cytoplasmic domains

cDNA coding for the A-domain of CopA was amplified from the CopA cDNA by PCR using primers 5'-ATGGGGGAGGCCATAAAGAAGCTCGTA-3' and 5'-GCCCATCGCGTCCTCGACCAGCTT-3'. This DNA was cloned into pBAD/TOPO vector, which introduces a carboxy terminal hexahistidine tag suitable for Ni^{2+} affinity purification, and transformed into *Escherichia coli* Top10 cells. The construct coding for other cytoplasmic domains were prepared as part of previous studies from our laboratory (González-Guerrero & Argüello 2008, Sazinsky et al 2006a, Sazinsky et al 2007, Sazinsky et al 2006b) (See Table I).

TABLE 1. CopA cytoplasmic domain constructs used in this study

Construct	Description	Tag	Reference
N-MBD	CopA fragment comprising amino acids M1-L77	Strep	Sazinsky et al., 2007
ATP-BD	CopA fragment comprising amino acids K407-K671	His	Sazinsky et al., 2006
A-domain	CopA fragments comprising amino acids G213-G326	His	This work
C-MBD	CopA fragment comprising amino acids L725–S804	Strep	González-Guerrero et al., 2008

2.2 CopA cytoplasmic domain preparation

Soluble domains expression and purification of CopA cytoplasmic domains were carried out as described (González-Guerrero & Argüello 2008, Sazinsky et al 2007, Sazinsky et al 2006b). Protein expression was induced for 3 h by the addition of 0.02% arabinose to cells containing pBAD-TOPO-based vectors or 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) to cells having pCRT7/NT-TOPO and pPRIBA1-based

plasmids. Cells were resuspended in 100 mM Tris (pH 8.0), 150 mM NaCl and disrupted using French Press at 15,000 p. s. i. Homogenates were centrifuged at 10,000 × g for 30 min. The resulting supernatants were centrifuged at 110,000 × g for 60 min. His-tag ATP-BD and A-domain were purified using a Ni^{2+}-nitrilotriacetic acid column and stored in 25 mM Tris (pH 8.0), 100 mM sucrose, 50 mM NaCl, and 10 mM ascorbic acid. Strep-tag N-MBD and C-MBD were purified by passage through a Strep-Tactin SuperFlow column (IBA). These proteins were stored in 100 mM Tris (pH 8.0), 150 mM NaCl, and 10 mM ascorbic acid at −80°C. Protein determinations were performed in accordance with Bradford (Bradford 1976).

2.3 Domain-domain co-purification assays using Ni^{2+} resin.

Interactions among cytoplasmic domains of CopA were studied by assessing their co-purification of isolated domains by batch affinity chromatography. 20 μM of His-tagged ATP-BD or A-domain and 40 μM of Strep-tagged N-MBD were incubated in a buffer containing 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 50 mM sucrose and 5 mM Dithiothreitol (DTT) (Buffer I) for 10 min at room temperature with gentle agitation. The effects of Cu^{+} and nucleotides on domain-domain interaction were investigated by using Cu^{+}-loaded N-MBD or including 5 mM ADP-$MgCl_2$ in the assay media. Cu^{+}-loaded N-MBD was obtained by incubating the protein with a five molar excess of $CuSO_4$ in 25 mM Tris-HCl, pH 7.5, and 10 mM ascorbic acid for 10 min at room temperature with gentle agitation. Unbound Cu^{+} was removed by passing through a Sephadex G-25 column (Sigma). Efficient Cu^{+}-loading was verified by measuring Cu^{+} content of the eluted protein using the BCA assay (Brenner & Harris 1995). The Cu^{+}-loaded N-MBDs was used immediately after removing unbound Cu^{+} to minimize Cu^{+} dissociation.

200 μM bathocuproindesidulfonic acid (BCS) was added to the reaction when Cu^{+} free conditions were required. Samples were incubated with 20 μl Ni^{2+}-nitrilotriacetic acid resin (Qiagen) for 10 min at room temperature and centrifuged at 14,000 rpm for 5 min to collect the unbound proteins in the supernatant (UP fraction). The proteins bound to the resin were washed with 200 μl 5 mM imidazole in Buffer I and 200 μl 20 mM imidazole in Buffer I, followed by elution with 50 μL of 150 mM imidazole in Buffer I (BP fraction). Protein content in the UP and BP fractions was analyzed by SDS-PAGE using 15% acrylamide gels and visualized by Coomassie Brilliant Blue staining (Laemmli 1970). Controls were performed where each protein domain was individually subjected to the same procedures; i.e., lacking the interacting partner.

2.4 Co-purification of interacting domains using StrepTactin resin.

Interactions were characterized as described above except that samples were incubated with 100 μl of pre-equilibrated Streptactin resin for 10 min at room temperature with gentle agitation. Then, they were centrifuged at 14,000 rpm for 5 min to collect the unbound proteins in the supernatant (UP fraction). The proteins bound to the resin were washed by 1 ml of 100 mM Tris pH 8.0, 150 mM NaCl (Buffer W), followed by elution with 500 μl of 2.5 mM desthiobiotin in buffer W (BP fraction). Protein content in UP and BP fractions was analyzed by 15% SDS-PAGE gel.

2.5 Co-purification of Na^{+}/K^{+}-ATPase ATPBD with CopA N-MBD.

The vector carrying the coding sequence for the His-tagged ATP-BD of the Na^{+}/K^{+}-ATPase was a gift of Dr. Craig Gatto (Illinois State University, Normal, IL) (Gatto et al

1998). Co-purification was performed as described above for the CopA ATP-BD and N-MBD.

2.6 Cross-linking of interacting cytoplasmic domains.

The cross-linking assay was performed as described with some modifications (Fancy et al 2000). Cross-linking reactions were carried out in a buffer containing 15 mM sodium phosphate (pH 7.5), 150 mM NaCl, and 0.125 mM Tris (2, 2'-bipyridyl) ruthenium (II) chloride. 20 μM of ATP-BD and 40 μM of N-MBD were used in the assay. Ag^+ was used as a substitute for Cu^+ since reducing agents interfere with the cross-linking reactions. Ag^+ bound N-MBD was obtained by incubating N-MBD with 10 molar excess of $AgNO_3$. N-MBD·Ag^+ was passed through Sephadex G-25 column to remove unbound Ag^+. Ammonium persulfate (APS) was added to final a concentration of 2.5 mM immediately before irradiation. The reaction mix was irradiated for 30 sec with a 100 W white lamp. Reactions were quenched with 10 μl of 100 mM Tris-Cl pH 6.8, 5 % SDS, 2 % β-mercaptoethanol, 20 % glycerol, 0.2 % bromophenol blue. Samples were subjected to SDS-PAGE and protein bands were visualized by electroblotting the gels onto nitrocellulose membranes and immunostaining with rabbit anti-His-tag polyclonal antibody and goat anti-rabbit IgG horseradish peroxidase-conjugated polyclonal antibody (GenScript, Piscataway, NJ).

3. Result

3.1 **The ligands dependent interaction between N-MBD and ATP-BD.**

Placing CopA in a nucleotide bound form appears required for full loading of metal transport sites by Ct-CopZ·Cu^{+} (González-Guerrero et al 2009). Crystallographic studies of Ca^{2+}-ATPases indicated a significant rearrangement particularly of cytoplasmic regions upon nucleotide binding (Olesen et al 2007) (Jensen et al., 2006; Toyoshima 2007; Olesen et al., 2007). The structure of CopA obtained by cryoelectron microscopy in the absence of ligands suggests the proximity of N-MBD to both ATP-BD and A-domain (Wu et al 2008). Then, does the ligand binding driven conformational change affect other aspects of the enzyme function, such as the interaction with regulatory domains (Tsivkovskii et al 2001)? The cytoplasmic domains (N-MBD, C-MBD, A-domain and ATP-BD) of *A. fulgidus* CopA can be expressed in isolated forms. These domains still maintain their structures and ligand (Cu^{+} or nucleotides) binding capabilities (Sazinsky et al., 2006; Mandal et al., 2004; Mandal et al., 2003; Mandal, et al., 2002; Sazinsky et al., 2006; González-Guerrero and Argüello, 2008; González-Guerrero et al., 2008). The putative interactions among these domains were examined by determining their co-purification by batch affinity chromatography when domain pairs carried alternative tags, either $(His)_6$- or Strep-tags. The structure of CopA obtained by cryoelectron microscopy in the absence of ligands suggests the proximity of N-MBD to both ATP-BD and A-domain (Wu et al 2008). Co-purification experiment indicates that the N-MBD does interact with ATP-BD (Fig. 7). Fig. 7 shows that Strep-N-MBD, upon incubation with His-ATP-BD, remained associated to Ni^{2+}-resin. However, when the assay was performed in the presence of Cu^{+}, no interaction between N-MBD and ATP-BD was

detected. Control experiments showed that absence of ATP-BD, Strep-N-MBD did not bind the Ni^{2+}-resin. Alternatively, assays performed co-purifying the N-MBD/ATP-BD complex using Streptactin resin yielded equivalent results in Fig. 8. Similarly, this interaction and its prevention by the presence of metal was observed in cross-linking experiments using $Ru(bipy)_3Cl_2$ and Ag^+ as substitute for Cu^+ (reducing agents interfere with the cross-linking reactions) (Fig. 9). It appears that the binding of Cu^+ to the N-MBD prevents the interaction with the ATP-BD.

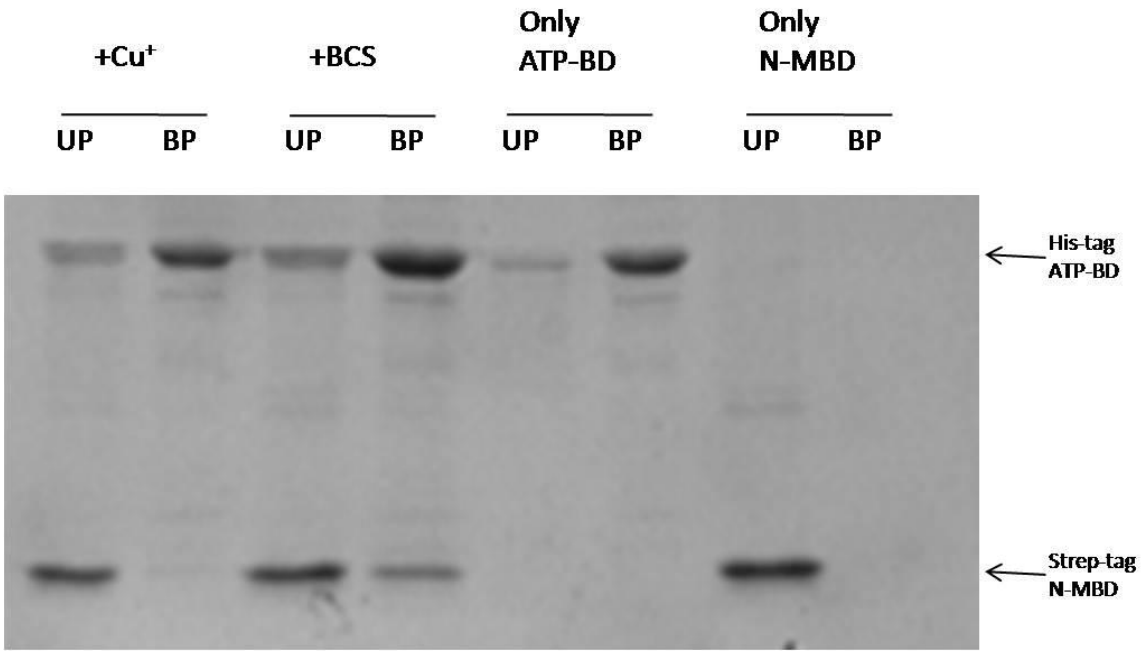

Figure 7. Cu^+ dependent interaction between N-MBD and ATP-BD. SDS-PAGE of a representative co-purification assay between ATP-BD and N-MBD or Cu^+-loaded N-MBD. 40 % of unbound protein (UP) or 40 % of the bound protein (BP) fractions were loaded in each lane.

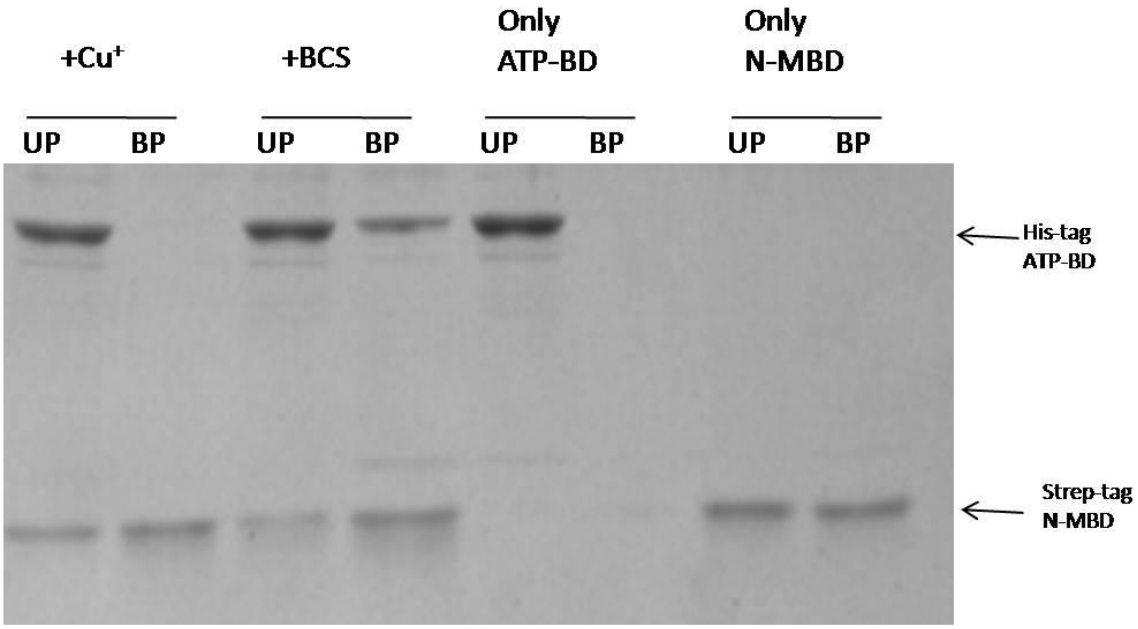

Figure 8. Cu^{+} dependent interaction assay between Strep-tagged N-MBD and His-tagged ATP-BD. SDS-PAGE of a representative co-purification assay using Strep resin. 40 % of unbound protein (UP) and 40% of bound protein (BP) fractions were loaded in each lane.

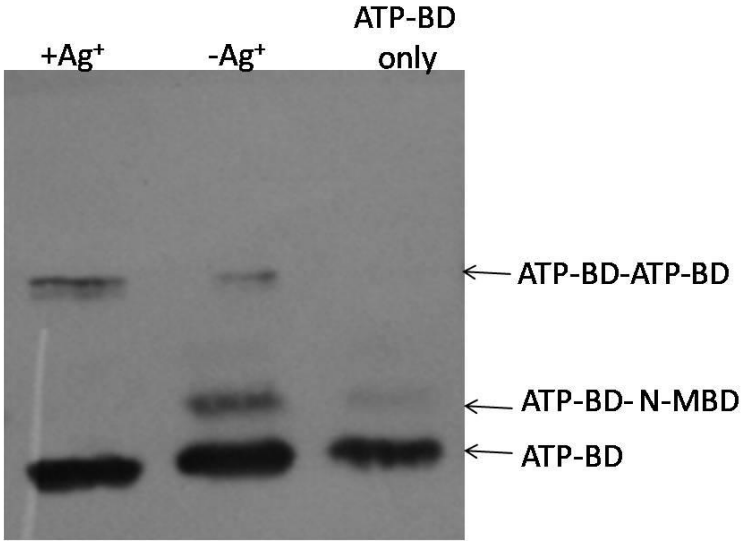

Figure 9. Cross-linking assay between Strep-tagged N-MBD and His-tagged ATP-BD.

Immunostaining of a cross-linking assay between ATP-BD and N-MBD or N-MBD ·Ag^{+}.

It was then interesting to investigate whether the binding of nucleotide to the ATP-BD would affect the interaction. Fig. 10 shows that the presence of saturating ADP prevented N-MBD/ATP-BD co-purification. ADP was used instead of ATP to avoid any slow ATP hydrolysis driven by the ATP-BD. These observations indicate that conformational changes driven by binding of substrate (nucleotide to ATP-BD) or regulatory ligand (Cu^{+} to N-MBD) affects the interaction among these domains.

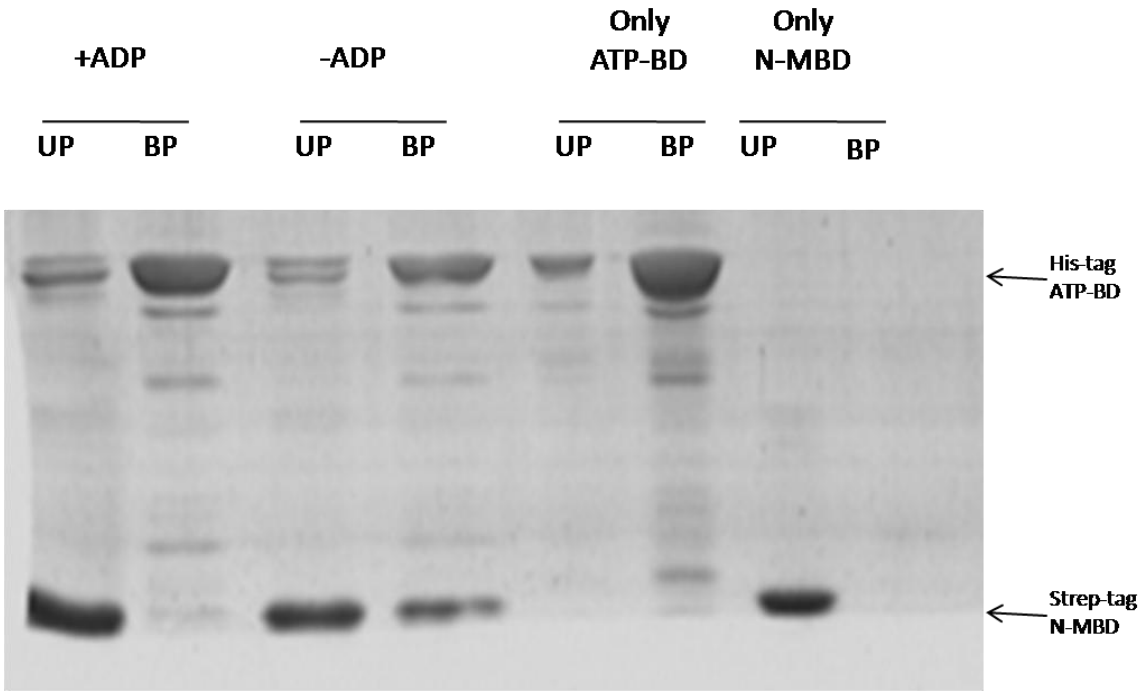

Figure 10. Nucleotide dependent interaction between N-MBD and ATP-BD. SDS-PAGE of a representative co-purification assay between N-MBD and ATP-BD in the absence and in the presence of 5 mM ADP-Mg^{2+}. 40 % of unbound protein (UP) or 40 % of the bound protein (BP) fractions were loaded in each lane.

The interaction between N-MBD and ATP-BD from Na^{+}/K^{+} ATPase was also tested by the co-purification assay. (Fig. 11) The result shows that the N-MBD could not bind to

ATP-BD from Na^+/K^+ ATPase. This result means that the association between N-MBD and ATP-BD from CopA is specific.

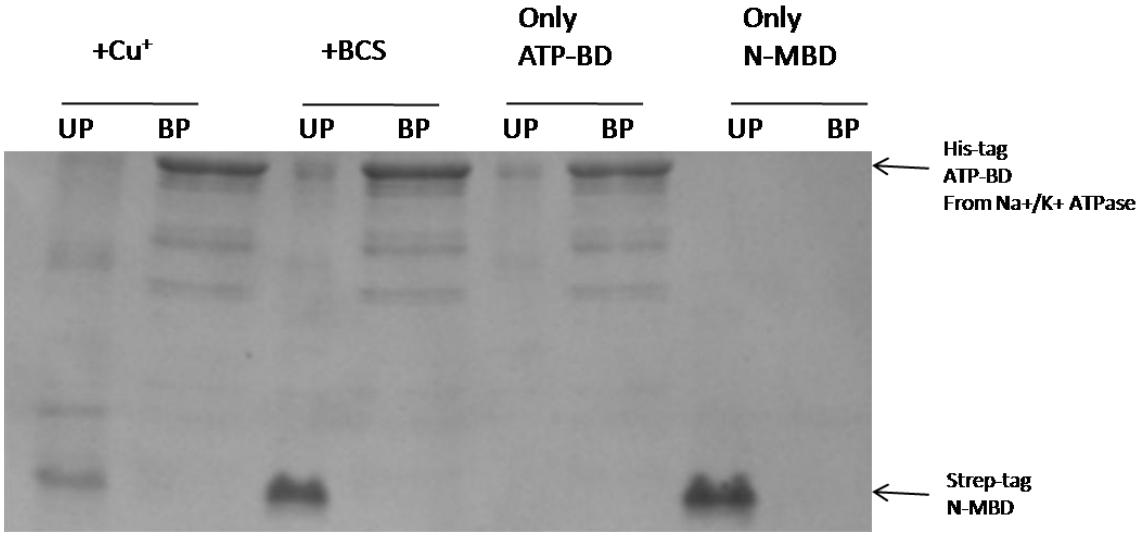

Figure 11. Interaction assay between N-MBD (20 µg) and Na^+/K^+-ATPase ATP-BD (49 µg). SDS-PAGE of a representative co-purification assay between Na^+/K^+-ATPase and either N-MBD or Cu^+-loaded N-MBD. 40% of unbound protein (UP) and 40% of bound protein (BP) fractions were loaded in each lane.

3.2 The N-MBD does not interact with A-domain.

The probable association of N-MBD with A-domain has been suggested by structural (Wu et al 2008) and biochemical studies (Hatori et al 2007). However, interaction among these domains was not observed in co-purification experiments (Fig. 12). Cross-linking experiments among these domains were prevented by the high homodomain interactions observed under all tested experimental conditions in conjunction with the similar molecular size of these two domains (not shown).

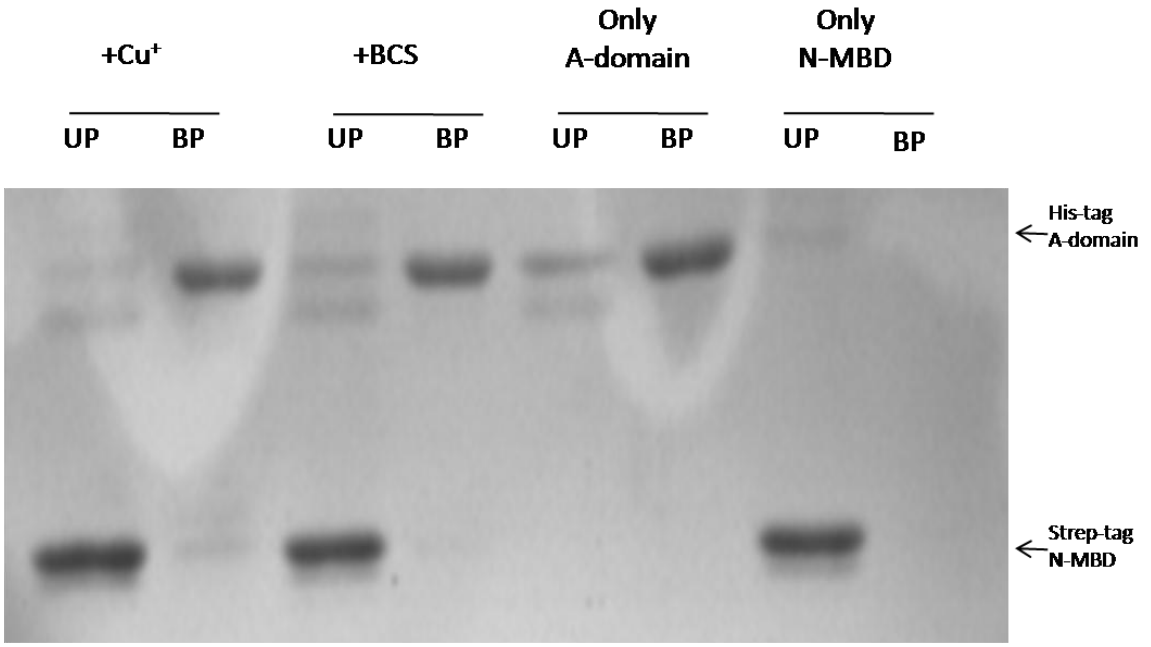

Figure 12. Absence of interaction between N-MBD and A-domain. SDS-PAGE of a representative co-purification assay between A-domain and N-MBD or Cu^{+}-loaded N-MBD. 40 % of unbound protein (UP) or 40 % of the bound protein (BP) fractions were loaded in each lane.

3.3 The ligands independent interaction between C-MBD and ATP-BD.

A. fulgidus CopA has a 7 kDa C-MBD structurally similar to N-MBDs and soluble Cu^{+}-chaperones. C-MBDs are not present in other Cu^{+}-ATPases although, alternative regulatory metal binding sites are found in the C-terminus of other heavy metal ATPases (Argüello 2003, Eren et al 2006). The C-MBD binds Cu^{+} with very high affinity thus it can stoichiometrically remove Cu^{+} from Cu^{+}-chaperones (González-Guerrero & Argüello 2008). However, mutation of its metal binding cysteines ($Cys^{751,754}Ala$) or removing the entire domain does not have significant effects on enzyme function (González-Guerrero & Argüello 2008, Mandal & Argüello 2003). Surprisingly, the C-MBD could interact

with the ATP-BD (Fig. 13). Moreover, different from the N-MBD, the presence of Cu^{+} or ADP in the assay media did not prevent these interactions (Fig.13, 14).

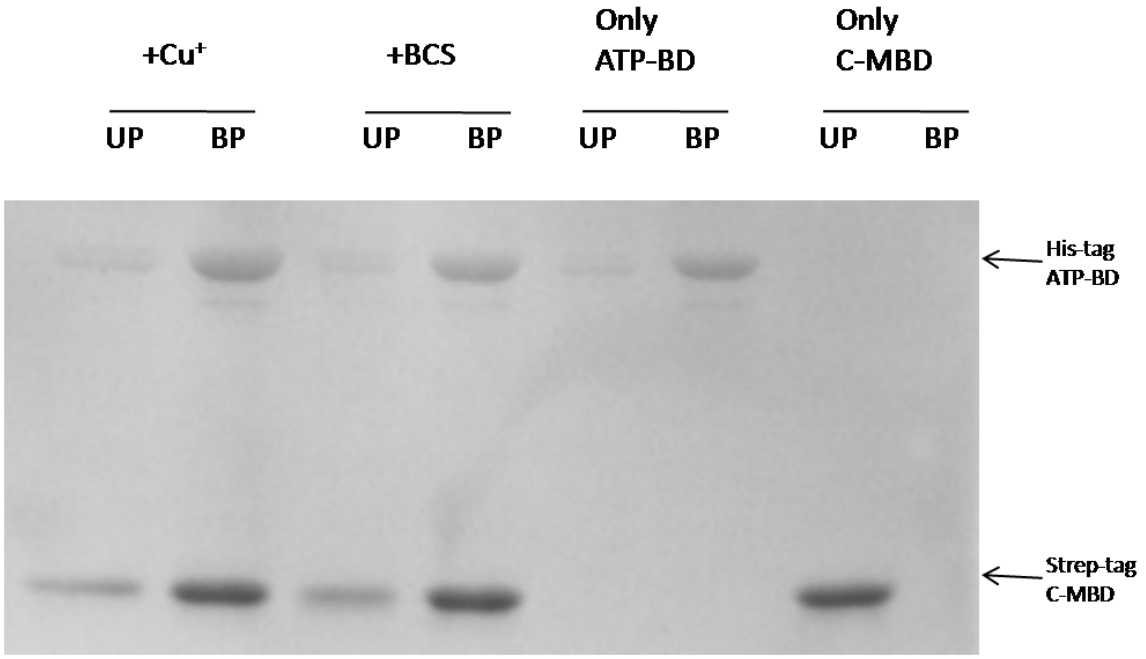

Figure 13. Cu^{+} independent interaction between C-MBD and ATP-BD. SDS-PAGE of a representative co-purification assay between ATP-BD and C-MBD or Cu^{+}-loaded C-MBD. 40 % of unbound protein (UP) or 40 % of the bound protein (BP) fractions were loaded in each lane.

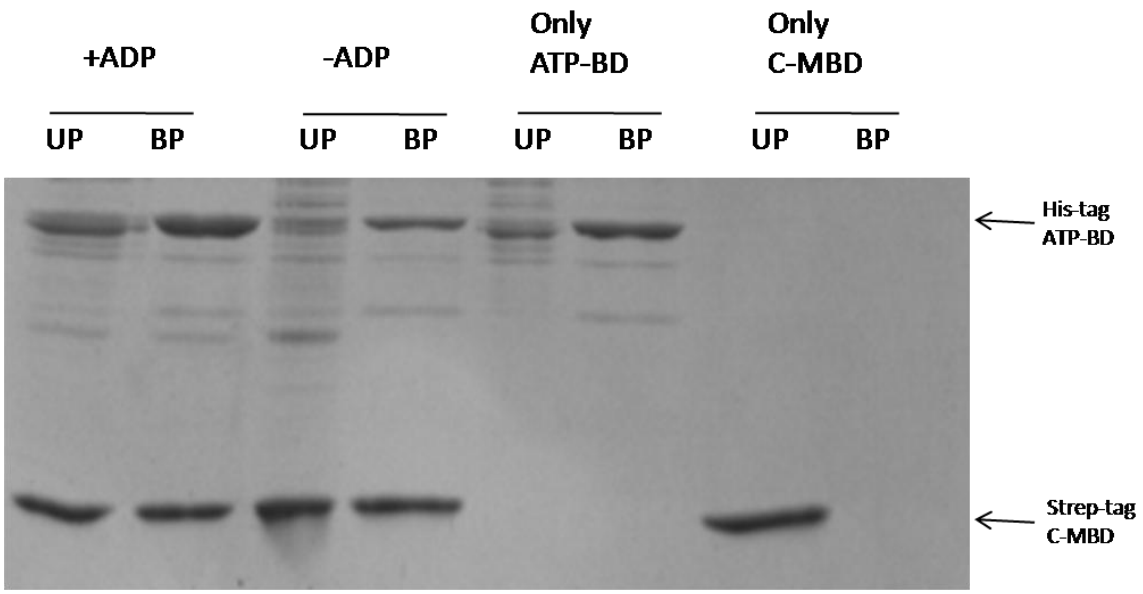

Figure 14. Nucleotide independent interaction between C-MBD and ATP-BD. SDS-PAGE of a representative co-purification assay between C-MBD and ATP-BD in the absence and in the presence of 5 mM ADP-Mg^{2+}. 40 % of unbound protein (UP) or 40 % of the bound protein (BP) fractions were loaded in each lane.

Is C-MBD bound to ATP-BD specific? Can it bind to ATP-BD of other P-type ATPases? The control experiment shows that the C-MBD still could associate with the ATP-BD from Na^+/K^+ ATPase in present or absent of Cu^+ (not shown). This result suggests that the interaction between C-MBD and ATP-BD is not specific, thus this interaction is not physiological relevant.

3.4 The Cu^+ independent interaction between C-MBD and A-domain

The relationship between C-MBD and A-domain was also tested. Interestingly, the result shows that the C-MBD still could interact with A-domain in presence or absence of Cu^+ (Fig. 15). This was surprising since the N-MBD which has the similar structure like C-MBD, did not interact with the A-domain.

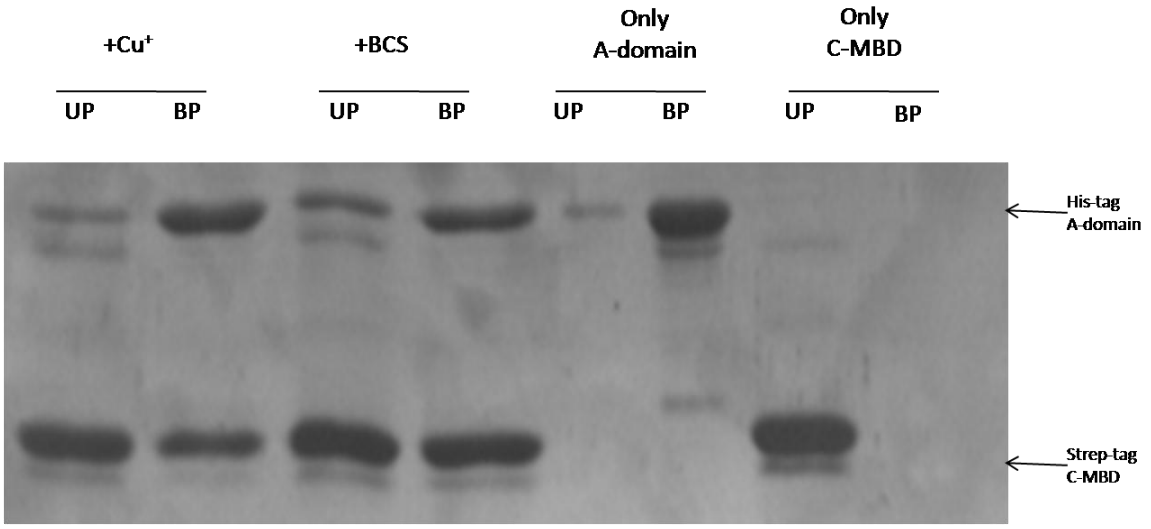

Figure 15. Interaction between C-MBD and A-domain. SDS-PAGE of a representative co-purification assay between A-domain and C-MBD or Cu^+-loaded C-MBD. 40 % of unbound protein (UP) or 40 % of the bound protein (BP) fractions were loaded in each lane.

Since C-MBD could interact with the ATP-BD, the A-domain and even the ATP-BD from Na^+/K^+ ATPase, we would doubt that these interactions are due to that C-MBD has special structural properties that could bind to all the other proteins. To eliminate that possibility, a control experiment with an unrelated protein (BSA) was performed. The result indicates that the association of C-MBD with ATP-BD and A-domain appears

nevertheless specific since it shows no co-purification with BSA (Fig. 16). Consequently, identification of its functional role remains elusive.

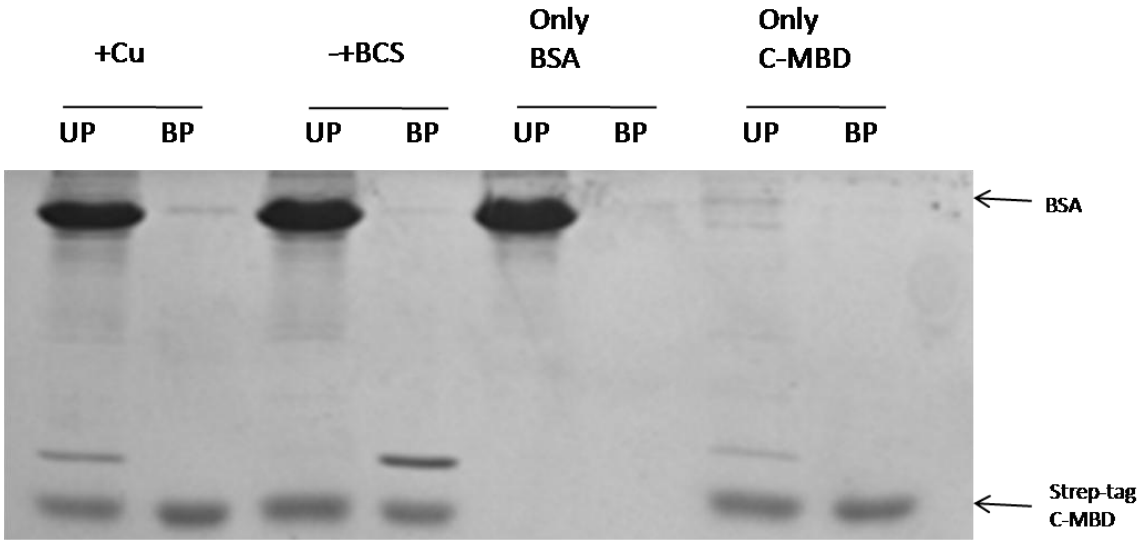

Figure 16. Interaction assay between Strep-tagged C-MBD and BSA. SDS-PAGE of a representative co-purification assay using Strep resin. 40 % of unbound protein (UP) and 40% of bound protein (BP) fractions were loaded in each lane.

4. Discussion:

Recent observations have indicated that Cu^+-chaperones directly transfer the transported Cu^+ to the corresponding ATPase TM-MBSs via protein-protein interactions guaranteeing that the metal is at no time released into the cytoplasm (González-Guerrero & Argüello 2008). Experiments testing equilibrium binding of free Cu^+ to the CopA have shown the presence of two TM-MBSs with fentomolar affinities for the Cu^+ (González-Guerrero et al 2008). In agreement with well-described mechanistic characteristics of P-type ATPases, both sites need to be occupied for catalytic enzyme phosphorylation and subsequent transport (Mandal et al 2004, Toyoshima 2008). However, chaperone-mediated metal transfer to the ATPase TM-MBSs under equilibrium conditions leads to loading of a single TM-MBS (González-Guerrero et al 2008). The recent findings in our lab show that an ADP induced conformational change will let the CopA accept the second Cu^+ form the chaperone CopZ (González-Guerrero et al 2009). The data reported here explain how ligands binding will affect the interactions between the cytoplasmic domains, and support a mechanism of Cu^+ transfer consistent with the transport requirements.

4.1 **Regulatory interaction between N-MBD and ATP-BD.**

Our data shows that the single N-MBD in CopA interacts with the ATP-BD. This brings additional experimental support to the CopA structure proposed by Wu et al. (Wu et al 2008). Cu^+ binding to N-MBD prevents this interaction in a manner similar to that previously described for the six N-MBDs present in the human ATP7B with its corresponding ATP-BD (Tsivkovskii et al 2001). However, our data hint at a specific role of the N-MBD proximal to the membrane in the proposed control of turnover kinetics

observed in all Cu^+-ATPases. In relation with the described effect of low affinity nucleotide binding on Cu^+ delivery to TM-MBSs, it is interesting that milimolar ADP also prevents the N-MBD/ATP-BD interaction. However, this effect does not exclude the regulatory role of Cu^+ binding to N-MBD, since this is observed in the ATPase activity of full-length proteins when measured at saturating nucleotide concentrations (Mandal & Argüello 2003).

Studies using limited proteolysis of *Thermotoga maritima* CopA have suggested the close proximity and likely interaction of its single N-MBD with the A domain (Hatori et al 2007). This was not observed when testing the co-purification of isolated domains. However, the different approaches and model proteins might be responsible for the apparent discrepancy.

4.2 The interaction with the non-functional C-MBD

C-MBD has the similar structure like N-MBD and Cu^+ chaperone and has the high affinity to bind the Cu^+. However, removal of the entire domain or mutation of its metal binding cysteines ($Cys^{751,754}Ala$) does not have significant effects on enzyme catalytic cycle and function (González-Guerrero & Argüello 2008, Mandal & Argüello 2003), 2003) Our results show that C-MBD undergoes a ligand independent interaction with both of the ATP-BD and A-domain and even the ATP-BD from Na^+/K^+ ATPase. Nevertheless the C-MBD could not interact with the unrelated protein (BSA). These findings indicate that the C-MBD has the specificity to the domains from P-type ATPases, but will bind to these domains all the time during the catalytic cycle. Thus, our results

also confirmed the previous finds that the C-MBD does not play any catalytic role in CopA.

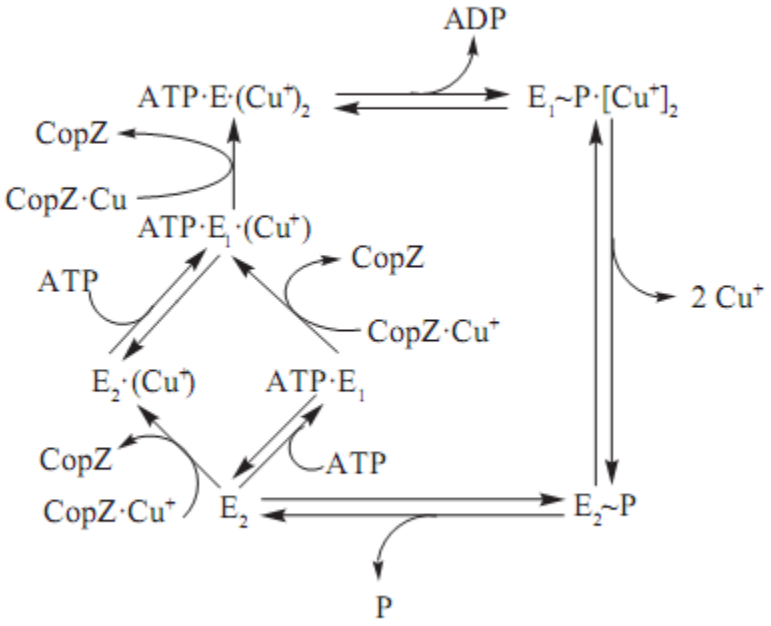

Figure 17. Proposed catalytic and transport cycle of Cu^+-ATPases.

4.3 Domain-domain interaction and catalytic cycle.

How does the domain-domain interaction relate to the CopA catalytic cycle? Combined our result with the fact that, the ATP bound to CopA will help the CopZ transfer the second Cu^+ to CopA TM-MBS, we suggest the new catalytic mechanism (Fig. 17).

Since the ATP could dissociate N-MBD from ATP-BD and ATP bound to CopA is essential for CopA receiving the second Cu^+, we could propose the two possible mechanisms. First is that the CopZ deliver Cu^+ to CopA, and then ATP bound to CopA changing CopA conformation to accept the second Cu^+ from CopZ? Or ATP bound to

CopA first, which will cause the enzyme undergo the big conformational change to accept the two Cu^{+} from delivered by CopZ. To discriminate among these paths is not possible at this point. In any case, the ATP bound to CopA will change the domain-domain interaction. The changing in the domain-domain interaction will cause the conformational change in CopA. The conformational change in CopA will help CopA receive the second Cu^{+} from CopZ. When both TM-MBSs are occupied, CopA starts to transport the Cu^{+} across the membrane.

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