

Structural-Functional studies of *A. fulgidus* CopZ Cu⁺

by

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ABSTRACT

Copper is an essential biological metal. Many enzymes such as cytochrome C oxidase and superoxide dismutase use copper as a prosthetic group. However, free copper can be harmful by generating reactive oxygen species through Fenton reactions (Linder and Hazegh-Azam 1996). Therefore, copper is tightly regulated throughout the cell. Cu^+ ATPases are transporters that regulate Cu^+ homeostasis. Mutations to these Cu^+ ATPases can cause diseases (Wilson's and Menkes diseases) (Ferenci 2005). CopA from *Archaeoglobus fulgidus* is a model Cu^+ ATPase; it contains eight transmembrane helices and a cytosolic ATP binding domain. CopA is assisted by a Cu^+ chaperone (CopZ) which we have shown delivers Cu^+ to CopA via protein-protein interactions (Guerrero-Guerrero and Arguello 2008). CopZ has two domains which are both able to bind Cu^+ (Guerrero-Guerrero and Arguello 2008). The N-domain has a mononuclear Zn^{2+} site, a [2Fe-2S] cluster and is extremely rich in cysteines (Sazinsky, LeMoine et al. 2007). To explore the role of cysteines and their Cu^+ binding capacity, each cysteine was mutated to serine. All resulting proteins were able to activate CopA ATPase. However, mutation of two Cysteines (Cys4 and Cys38) led to binding of two additional Cu^+ ions. Since these two Cys participate in Zn^{2+} coordination we hypothesized that the coordination of Cu^+ might take place with the displacement of Zn^{2+} . In order to explore this hypothesis, CopZ-WT was incubated with an excess amount of Cu^+ , followed by atomic absorption to measure the presence of Zn^{2+} . These experiments demonstrate a displacement of Zn^{2+} by Cu^+ . Thus, our results show that the second Cu^+ binding site in *A. fulgidus* CopZ is constituted by the same Cysteines participating in Zn^{2+} binding.

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LIST OF ABBREVIATIONS

AAS: Atomic Absorption Spectroscopy

ATP: Adenosine Triphosphate

ATP-BD: ATP Binding Domain

A-Domain: Actuator domain

BCA: Bicinchoninic acid

C-MBD: C-terminus Metal Binding Domain

CopZ-WT: Wild Type CopZ

CopZ-CT: CopZ C-Domain

CopZ-NT: CopZ N-Domain

DTT: Dithiothreitol

IPTG: Isopropyl β -D-thiogalactopyranoside

MBD: Metal Binding Domain

N-MBD: N-Terminus Metal Binding Domain

N-Domain: Nucleotide Binding Domain

P-Domain: Phosphorylation Domain

Pi: Inorganic phosphate

PMSF: Phenylmethylsulfonyl fluoride

TCA: Trichloroacetic acid

TM: Transmembrane

1. INTRODUCTION

1.1 Cu Homeostasis

As a trace element, only ~100 mg of copper are present in adult humans (Linder and Goode 1991). Nevertheless, copper is an essential element in biological systems. Many enzymes such as cytochrome c oxidase and superoxide dismutase use copper as a prosthetic group. It has been estimated that over a quarter of all known enzymes require a particular metal ion for function (Horton, A. et al. 2002).

For example, in superoxide dismutase copper undergoes oxidation from Cu^+ to Cu^{2+} in order to convert two superoxide anions to O_2 and H_2O_2 at which point catalase will decompose H_2O_2 into H_2O (book; Brewer 2008). When bonded to superoxide dismutase copper is able to be used as a cellular antioxidant (Horng, Cobine et al. 2004).

Copper also plays a critical role in oxidative phosphorylation. Cytochrome c oxidase (also known as complex IV) contains two copper ions, which allow the successive transport of electrons through oxidation-reduction reactions and thereby releasing free energy to produce ATP (book; Brewer 2008). The biological and physiological importance of copper also extends to connective tissue formation (lysyl oxidase) and iron metabolism (ceruloplasmin) (Linder and Goode 1991).

The proper trafficking of copper is also extremely important. Wilson's disease and Menkes syndrome are caused by an inability to properly transport copper throughout the human body. ATP7B is a copper transporter expressed in hepatic tissue. The liver is involved in the clearance of excessive amounts of copper and is one of the major regulators of copper

homeostasis (Wimmer, Herrmann et al. 1999; Madsen and Gitlin 2007). Using the energy from ATP hydrolysis, ATP7B transports copper out of the liver and into bile, at which point copper is expelled out of the body through feces (Vonk, Wijmenga et al. 2008). Wilson's disease is caused by mutations in the ATP7B gene. Due to these mutations, copper is not able to exit the cell, which results in an over accumulation of copper in hepatic tissue (Ferenci 2005; Mak and Lam 2008). Menkes syndrome is caused by mutations in the copper transporter ATP7A. ATP7A is found in most tissues, but is absent from the liver. In the small intestine, ATP7A helps control the absorption of copper from food (Barnes, Tsivkovskii et al. 2005; Mak and Lam 2008). However, when ATP7A is not working properly, copper is unable to be exported out of the intestinal tissue, thereby causing a hyper-accumulation of copper in the intestinal tissue which leads to copper deficiency in peripheral tissues and associated copper dependent enzymes (Ferenci 2005). Side effects of these mutations include neurological damage, psychiatric symptoms, mental deterioration, liver disease, and, if not treated, death (P Ferenci 1996; Madsen and Gitlin 2007; Mak and Lam 2008).

Although trace amounts of copper are needed to perform biological functions, an excess of copper is toxic to the cell as free copper may generate free radicals. For example, interaction of free copper with H_2O_2 can produce $OH \cdot$ which can damage cells (Halliwell 1985; Brewer 2008). Copper is tightly regulated by the coordinated action of copper chelating agents, copper transporters and copper chaperones (Rensing, Ghosh et al. 1999; Gatti, Mitra et al. 2000). Due to these copper homeostasis mechanisms less than 0.2 pM of free copper is present in the cell (O'Hallorantta 2000; Brewer 2008).

1.2 P-type ATPases

P-type ATPases are membrane proteins that transport ions against their concentration gradients (Arguello and Eren 2007). Ions such as H^+ , Na^+ , K^+ , Cu^+ , Cu^{2+} , Ca^{2+} and Mg^{2+} are transported across the membrane through ATP hydrolysis. ATPases are involved with a diverse range of biological systems such as, action potentials in nervous tissues, Ca^{2+} -dependent signal transduction, secretion and reabsorption of solutes in the kidneys, acidification of the stomach, nutrient absorbance in the intestines and relaxation of muscles (Axelsen and Palmgren 1997; Cobine, George et al. 2002). As shown in Fig 1, P-type ATPases are grouped based on their putative ion specificity. The first group of P-type ATPases known as P_1 , contains P_{1A} (a bacterial Kdp ATPase) and P_{1B} ATPase which transport heavy metals such as Cu^+ and Zn^{2+} . The second group known as P_2 , contains the Na^+/K^+ ATPases, Ca^{2+} ATPases and H^+/K^+ ATPases. A third group, known as P_3 , contains the H^+ ATPases and Mg^{2+} ATPases. The fourth group of P-type ATPases known as P_4 , are responsible for transporting lipids. The substrate of the fifth group, known as P_5 , has not yet been defined (Axelsen and Palmgren 1997).

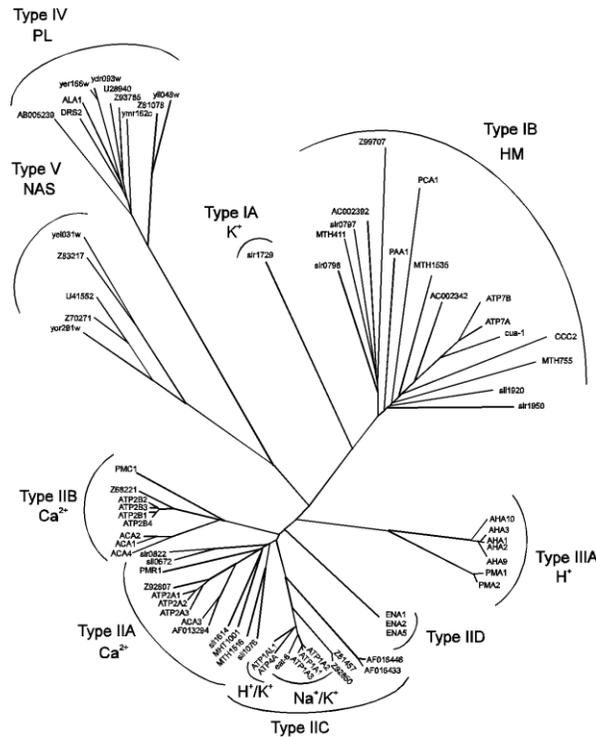


Fig. 1. Phylogenetic tree of P-type ATPases (Taken from Axelsen and Palmgren, 1998)

The topologies of P-type ATPases show six to ten transmembrane (TM) α helices and several of cytosolic domains (Fig. 2). The unifying characteristic of all P-type ATPases is the cytosolic domain known as the ATP binding domain (ATP-BD), which possesses the conserved DKTGT sequence. The aspartic acid residue located in this sequence is phosphorylated and causes the conformational change for the transport of ions across the membrane (1999).

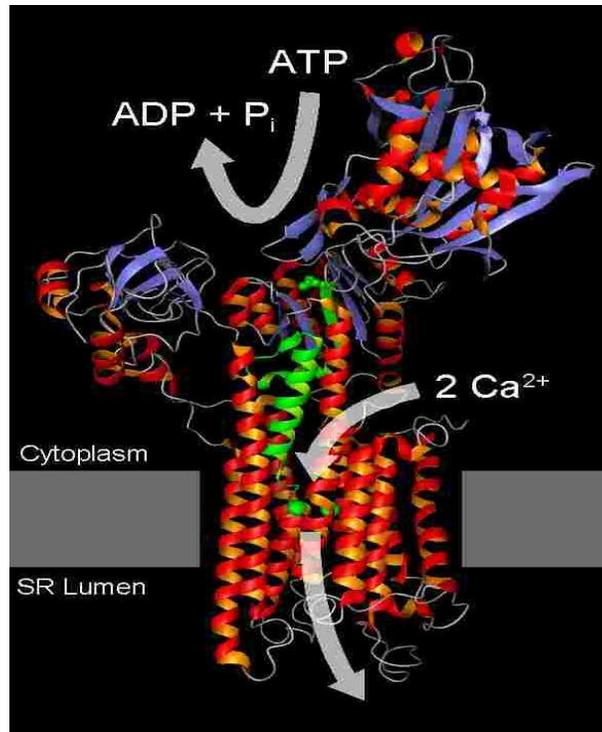


Fig. 2. Structure of the calcium ATPase according to Toyoshima et al. (Taken from Toyoshima et al. 2000)

All P-type ATPases follow the Post-Albers catalytic cycle. The enzyme shifts from the E1 to E2 conformation in order to transport ions into the extracellular spaces (Brooks and Lloyd Mills 2006). The enzyme then shifts back to the E1 conformation closing access to the lumen (Change 2001). Fig. 3 shows this catalytic cycle. Starting with step 4, ATP binds with high affinity to the cytosolic ATP binding domain, allowing the ion to bind to the transmembrane site. This forms the ATP.E1.nM^{n+} complex. In step 1, ATP is hydrolyzed to ADP forming a high energy phosphate bond which then forms complex $\text{E1P.(M}^{n+})_n$. Given that the phosphorylation state favors the formation of E2, the E1 complex $\text{E1P.(M}^{n+})_n$ is quickly converted to the E2 state. In the E2P complex the affinity of ion decreases resulting in a release of the ion into the extracellular/luminal. Once phosphate is released, the E2 conformation is formed and this completes the cycle (Change 2001).

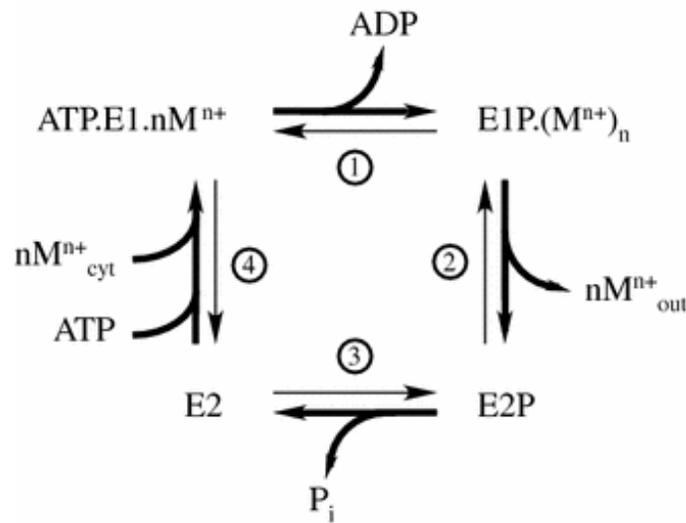


Fig. 3. P_{1B}-ATPases catalytic cycle. E1, E2, E1P and E2P represent the basic conformations that the enzyme can assume. Mⁿ⁺ represents a metal transported by these enzymes. n indicates the uncertainty on the specific stoichiometry of transport. Mⁿ⁺_{cyt} and Mⁿ⁺_{out} represents the cytoplasmic or extracellular/luminal localization of the transported metal (Taken from Argüello et al. 2003)

1.3 P_{1B}-type ATPases

P_{1B}-type ATPases, also known as heavy metal ATPases, transport heavy metals such as Zn²⁺, Cu⁺, Cu²⁺, Co²⁺ and Pb²⁺ through ATP hydrolysis (Fig. 4). Unlike other P-Type ATPases subfamilies, P_{1B}-type ATPases are found in all life kingdoms and have a distinct structure characterized by a reduced number of transmembranes (TMs), a smaller ATP binding domain, and the presence of N- and C-terminal metal binding domains (MBDs) (Arguello and Eren 2007). As shown in Fig. 4, most P_{1B}-type ATPases have a CPX signature sequence located in their TM H6. P_{1B}-type ATPases were first named CPX-ATPases due to the consistency of amino acids located on TM H6. Replacement of cysteine in the CPC of *E. coli* CopA resulted in the loss of metal transport (Fan and Rosen 2002). In our lab, replacement of these cysteine in *A. fulgidus* CopA still allowed for the binding of metal to transmembrane metal binding site but, resulted in

an inability to phosphorylate in the presence of copper and ATP (Mandal and Arguello 2003). Metal specificity for P_{1B}-type ATPases is determined by amino acids positioned on the last three transmembrane helices as seen in Fig. 4. Studies have shown that any mutation done to these amino acids results in the inability to transport ions (Mandal, Yang et al. 2004).

Most P_{1B}-type ATPases have cytosolic MBDs which are approximately 60-70 amino acids long. The MBDs contain a highly conserved CXXC consensus sequence which binds metal and adopts a $\beta\alpha\beta\beta\alpha\beta$ fold that is homologous to the structure of copper chaperone proteins such as human Atox1, yeast Atx1 and prokaryote CopZ (Mandal and Arguello 2003). The N-MBD plays a self-inhibitory role, by reducing enzyme activity. This is supported by experiments that were done in our lab to truncate the N-MBD from CopA. While the enzyme was still fully functional, the turnover rate greatly increased (Guerrero-Guerrero and Arguello 2008). Further studies in our lab have shown that the N-MBD, A-domain and ATP-BD interact with each other (Hong 2009). We speculate that the main function of the N-MBD is to prevent the rotation of the A-domain by working as a gate; when the N-MBD makes contact with the A-domain, it allows for the ATP-BD to stay inactive. However, when the N-MBD binds copper it releases contact with the A-domain, allowing ATP to bind to the ATP-BD and ion to bind to the transmembrane site at which point the ion can be transported out of the cell (Arguello and Gonzalez-Guerrero 2008). Since the N-MBD has a self-inhibitory role some P_{1B}-type ATPases do not have an N-MBD while others may have as many as six. The C-MBD of *A. fulgidus* CopA is the only known P_{1B}-type ATPase that contains this cytosolic domain, however, the C-MBD appears to have no functional role.

The ATP-BD is found between transmembrane H6 and H7 and is subdivided into two domains, the nucleotide binding domain (N-domain) and the phosphorylation domain (P-

domain). The P-domain contains the well conserved sequence DKTGT. As stated previously, the aspartic acid residue located in this sequence is phosphorylated and causes the conformational change facilitating the transport of ions across the membrane (1999).

The actuator domain (A-domain) is found between TM H4 and TM H5. In regards to CopA, this region bears little sequence similarity to the corresponding region in other P_{1B}Type ATPases. However, due to the presence of a highly conserved TGE (P/X) sequence and length it has been accepted as equivalent to the A-domain of P₂-type ATPases (Sazinsky, Agarwal et al. 2006).

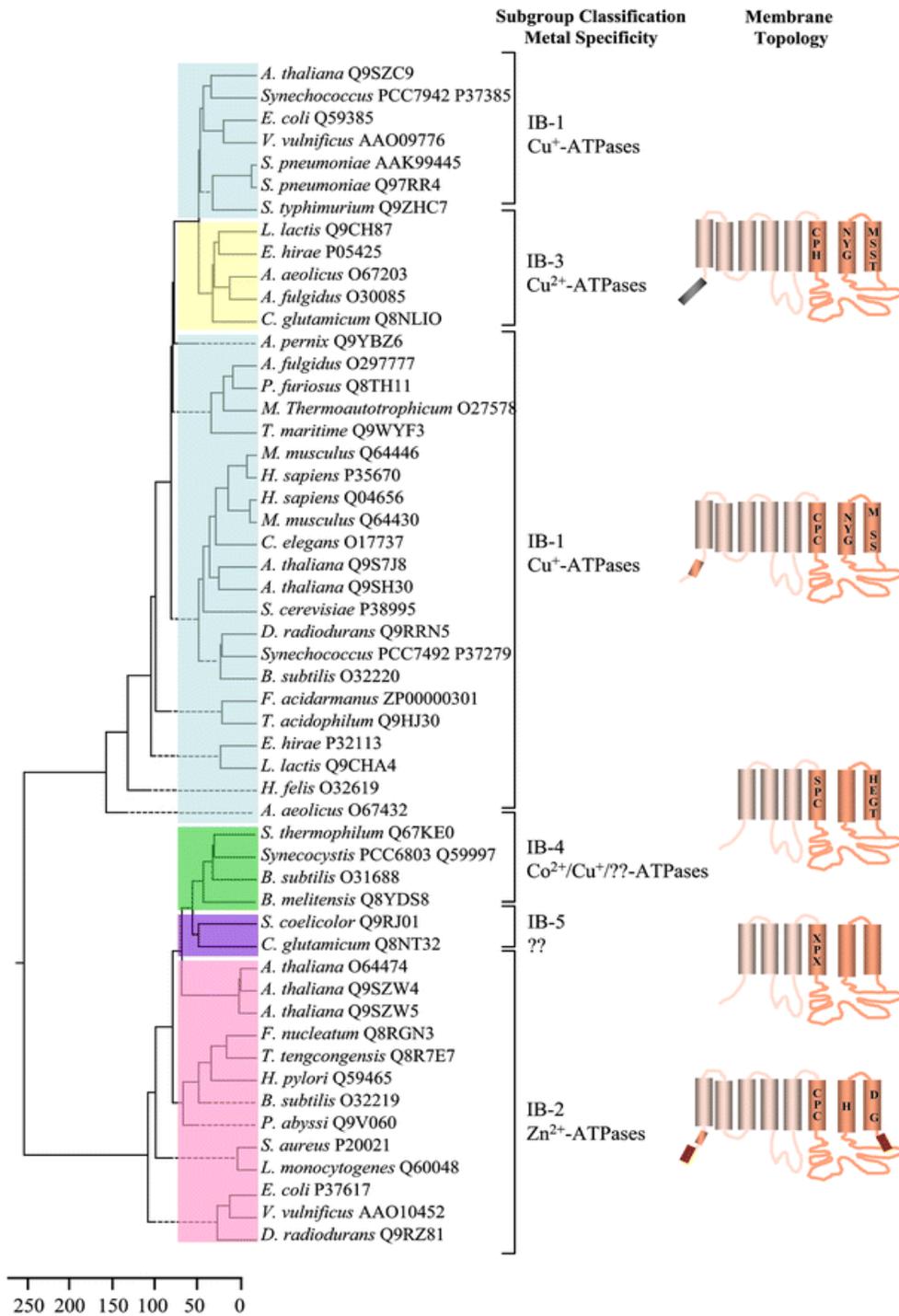


Fig. 4. Phylogenetic tree of the P_{1B}-type ATPases. The tree was prepared from a Clustal W alignment of representative sequences of P_{1B}-type ATPases. The relative abundance of sequences from each subgroup has been maintained. The metal specificity and the structural characteristics are indicated next to the subgroup denomination. Amino acids in TMs proposed to participate in determining metal selectivity. Black blocks represent His-rich N-MBDs; orange blocks, CXXC N-MBDs; and red, His and Cys rich N- and C-MBDs (taken from Argüello et al. 2003)

1.4 *Archaeoglobus fulgidus* CopA, a P_{1B}-type ATPase

CopA is a Cu⁺ ATPase present in the hyperthermophilic archaea, *A. fulgidus*. Due to the inherent thermo-stability of CopA, it is used in our lab as a model system for understanding P_{1B}-type ATPases such as the human ATP7A and ATP7B. During transport Cu⁺ is coordinated by these six amino acids located in the transmembrane metal binding site as indicated in Fig. 5. CopA has four cytosolic domains N-MBD, C-MBD, A-domain and ATP-BD (Mandal, Cheung et al. 2001). CopA is able to transport the reduced form of copper. Studies have shown that CopZ, the metallochaperone of CopA, is able to reduce Cu²⁺ to Cu⁺ to allow binding to the transmembrane metal binding site of CopA (Sazinsky, LeMoine et al. 2007).

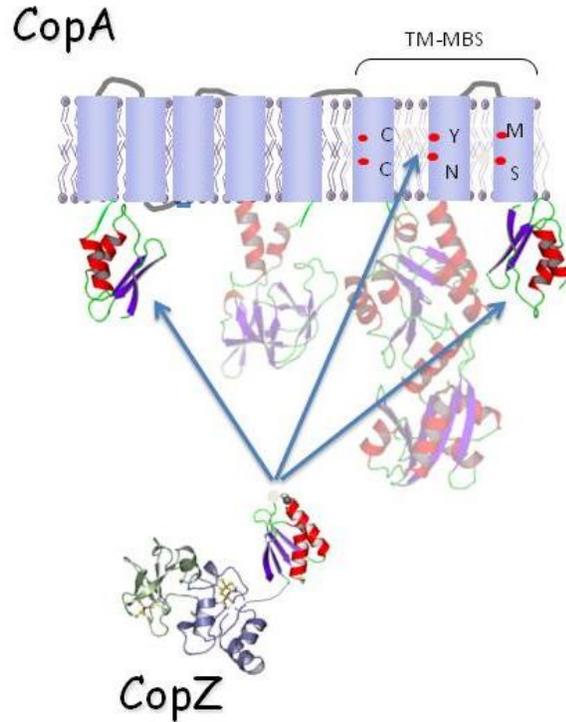


Fig. 5. Scheme of CopA and CopZ structures and hypothetical models for Cu^+ delivery to TM-MBS. The topological scheme of CopA includes the A-domain [Protein Data Bank (PDB) ID code 2HC8] and ATP-BD (PDB ID code 2B8E) structures. N-MBD and C-MBD structures are homology models based on the fourth N-MBD of the Menkes protein (PDB ID code 1AW0) and *Bacillus subtilis* CopZ (PDB ID code 1K0V), respectively. Amino acids involved in the Cu^+ coordination by the TM-MBS are indicated. CopZ scheme includes the N-CopZ structure (PDB ID code 2HU9) and the C-CopZ homology model based on *Enterococcus hirae* CopZ (PDB ID code 1CPZ). The green continuous line represents the direct delivery of Cu^+ by CopZ to TM-MBS. The red dotted lines symbolize the Cu^+ transfer from CopZ to N-MBD and C-MBD and the subsequent relocation of Cu^+ from the MBDs to the TM-MBS (taken from González-Guerrero et al. 2008)

1.5 Metallochaperones

Before the discovery of metallochaperones it was postulated that copper enzymes obtained their essential cofactors from free copper ions or complexes of copper with low-molecular-weight ligands, such as glutathione (Arnesano, Banc et al. 2002). Studies later revealed that very little free copper is available in the cytoplasm of eukaryotic cells (Brewer

2008). This exhibited the overcapacity of copper chelation and suggested more complex mechanisms for intracellular metal trafficking. Metallochaperones are a group of cytosolic proteins that bind tightly to a metal and ferry the metal to a specific location. The target may be a protein that requires this specific metal for function or a location in which the metal will be further transported across the membrane (Singleton and Brum 2007).

In 1997, the yeast ATX1 gene from *Saccharomyces cerevisiae* was shown to encode a metallochaperone. Since that time, many functionally homologous metallochaperones have been identified in bacteria (Cobine, Wickramasinghe et al. 1999), plants (Himmelblau, Mira et al. 1998) and several mammals (Nishihara, Furuyama et al. 1998). ATX1 is ~70 amino acids in length and has a highly conserved CXXC consensus sequence which binds copper. ATX1 also adopts a $\beta\alpha\beta\beta\alpha\beta$ fold which is found in a variety of proteins that bind inorganic ions (Rosenzweig and O'Halloran 2000; Arnesano, Banci et al. 2001). A characteristic of metallochaperones is the similarity in protein folding between the chaperone and the target enzyme (12). ATX1 binds Cu^+ and delivers it to Ccc2, a P-type ATPases that transports Cu^+ into trans-Golgi vesicles for incorporation into the multi-copper oxidase Fet3 (Singleton and Brum 2007).

The human metallochaperone Atox1 is homologous to ATX1 and also delivers copper in the reduced state to ATP7A and ATP7B (Hung, Casareno et al. 1998; Hamza, Faisst et al. 2001). Atox1 is 68 amino acids in length and contains both a CXXC binding motif and a $\beta\alpha\beta\beta\alpha\beta$ fold. Studies have shown that human Atox1 can substitute for yeast ATX1 in *atx1* Δ yeast mutant (Hung, Casareno et al. 1998). A crystal structure obtained of copper bound Atox1 is available (Wernimont, Huffman et al. 2000). The crystal structure revealed that human Atox1 has a dimeric shape. The bound copper was coordinated in a distorted tetrahedral shape with three cysteine residues (Wernimont, Huffman et al. 2000; Muller and Klomp 2009)

1.6 Archaeoglobus fulgibus CopZ

CopZ is a metallochaperone present in the archaea hyperthermophilic *A. fulgidus* which ferries Cu^+ to CopA for transport (Fig. 6.) (Guerrero-Guerrero and Arguello 2008). This metallochaperone has two domains a C-domain (CopZ-CT) and an atypical N-domain (CopZ-NT) both domains are able to bind one Cu^+ . CopZ-CT is 73 amino acids in length and also contains the conserved sequence CXXC metal binding motif and a $\beta\alpha\beta\beta\alpha\beta$ fold which are both very characteristic of metallochaperones (Hung, Casareno et al. 1998; Sitthisak, Knutsson et al. 2007). However, CopZ-NT differs greatly from other metallochaperones. CopZ-NT is homologous to proteins found only in extremophiles and is the only such protein that is fused to a copper chaperone (Fig. 7A) (Sazinsky, LeMoine et al. 2007). “In all other extremophilic organisms that have a CopZ-NT homolog, the putative copper chaperone exist as a separate 70 amino acid protein, and its gene is not located in an operon with that encoding a CopZ-NT homolog, suggesting that their expression might not be linked” (Sazinsky, LeMoine et al. 2007). The C-domain of CopZ shares a 42% identity with the C-MBD of CopA and the N-domain of CopZ shares only a 20% identity with the N-MBD of CopA (Sazinsky, LeMoine et al. 2007).

CopZ N and C terminal Domain

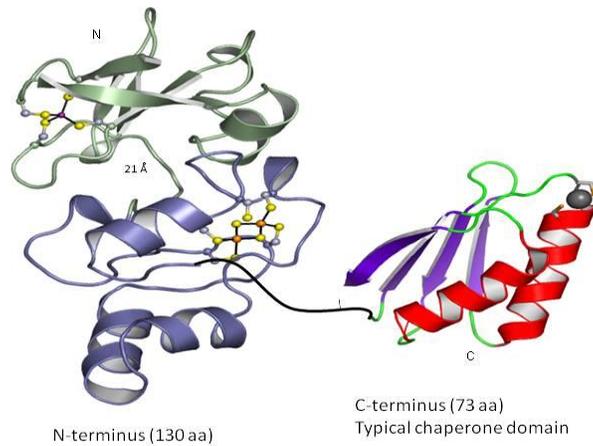


Fig. 6. Structure of N-terminal domain resolved by X-ray diffraction
Structure of C-terminal domain obtained by homology modelling.
(Taken from Sazinsky et al. 2007)

A.

Atox1	1	---	MPKHEFSVDMT	CGGCAE	AVSRV	LNLK	GGVKY	DIDL	PNKKV	CIESEH	SMDT	LLATL	KKTG	KTVS	VLGLD	---	72																			
CopA-NT	12	---	ERTV-RVT	GMTCAM	CVKSI	ETAV	GSLE	GVVE	VRVNL	ATETA	FIRF	DE	KRID	FETIKR	VI	EDL	CGVV	DQAA	86																	
CopZ-CT	133	KA	KRVEIK	DGLT	CMGC	SAV	AALE	EA	-GAN	VVEI	GLD	--RA	VVE	DE	--AL	QL	VE	VEG	GYS	RI	LE	KR	202													
CopA-CT	731	DS	VEKIV	LE	SG	SCH	HCA	RV	KA	LE	EA	-GR	VEK	VD	LN	--EA	V	AG	NK	--D	V	D	Y	K	A	V	E	A	G	Y	O	A	K	R	--	802

B.

	Cys Rich Domain	Mononuclear Zn/Fe Domain	
Archaeoglobus	-----MAINNECCGGKKER-----	-----MNRCEGSTEGRWLPPLTVGAHVKEGLWSK--IKGDFYFCSLESCEVVYFN--EQT	52
Alkaliphilus	-----LNQIKEKCPVCSNEGIAVSKVTVHEHLVAD--NYRNVIDGGQYKICINEDODVVYSLDNEI	74	
Caldicellulosiruptor	-----MDCCFLSNFSCCESSY-----	-----IETPSQTCPICKHQGILVKNFVVKHIVLD--EYLPQIGINDYFLCTNPFCDVGHYQGEN--	73
Geobacillus	-----MDCCAPTNRGETTET-----	-----NCPKCGQNGRRVPLLTLSLLKPAALATLVPDRDYVFCPNPFCEETVYFALDGS	69
Thermosynechococcus	-----MDCCGYPEPTEGDSF-----	-----MEMPRDRPQHCQDQSQGKPKLTLTKSLLIPQALERLNSQASRYFCSSPDCPIVYFATAED--	78
Deinococcus	-----MTGTPFLEVHPATVRLDPRRVYGWGMAASSPAVPCPVGSHASLPVRLTLKALLRPQALARLQPASDFRCPDPAC	86	
Chloroflexus	-----MTTPECSPTESATCGLNTP-----	-----RRQTVSPVCGASGKRVGLQTVKALVS--ISLRVQVGSFWRQSQHCPVVYFAADNGQ	77
Moorella	-----MGLEICGSEHGSS-----	-----AKPCECGTVGKRVAGITVASLLN--TAVREKISDTQYNLCLSPCKVVYVYGDGDS--	68
Thiomicrospira	-----MSCCSTSDSCSKRAG-----	-----KLTCPVNNKLYQSVSPLTVLHNLAA--PWKNFSAEVTYVYFDDPDCVVIYFSDSGS--	71
Carboxydotherrmus	-----MNQDRMNQDRCSCCDG-----	-----MNCPCGTEGKIVFAETVFNLLKDEEKERFQDG--LYPACTNPSCPVAYFG--E	51
Wolinella			
Thermoanaerobacter			
Chlorobium			16

	2Fe-2S Domain	
Archaeoglobus	VFRKGLKTRVGVKER-EEPKPVYCNRVTEKMLLEAAEKFGKEK---AVEITGAGK-GKWCVVNPSGRCCWHHLERLGFVPGGE	132
Alkaliphilus	KFLRDQIRVPIWFKKD-AEPKYACYGSGVTEQDIIIEAVVYGVKTVKEVNMITGAMK-NSNCKEKNPLGICCHKTQQAIYRGLAL	155
Caldicellulosiruptor	VFYRQQLKVPVWLKDD-ANPKYVYCGKVTEDIMDAVLKKGAKT-LSEACKITGAMK-DCRCEINNPFGKCAAUVKEAFERATRI	157
Geobacillus	AFFVGDLLKVPVFKDS-AMDVPVYCFDWTREIRIAEAVRHGGEP--LQEIKAHVQAGR--CGCEVNNPQAGCCMGNVAEIRQVNGN	150
Thermosynechococcus	TFTTDLKVPVFKNP-REEVVPYCFGWTRQLQHLGQEQPLAIAAITAHIQAGR--CGCEVNNPQSGCCLEDVRYVVEAAGA	161
Deinococcus	-FRVPDLKVPVAHKDP-SGGVPLCYCFGTRAEVEAAEAGRAELVETIRGHIRAGR--CGCEVNNPQSGCCCLGMVSGLLASP---	165
Chloroflexus	TLTVDQIRERVYQKEPERPDVLCYCFQYRVGDIQQAS-PEARAAVIADIKAGIAADQ--CACDVRNPQSGCCCLGNVQQVAKVQKLT	160
Moorella	LFTRDQVRVPWFKEQ-ASPRIICYCKNVTDSDILEHVVTTRQCCNSLQDIRKHTRANT-GHECLTKNPAGS-----	136
Thiomicrospira	IFKRPDIRSLFNKTKADTNDLICYCFVSKTDYLDNPGTR-----EFVVEQTKLKH--CACEVQNPQSGCCCLKDFPKGS-----	143
Carboxydotherrmus	TFEVDLIKTTLWYKDE-GDDVLYCYCNKVSREIKKAFRQVGPD--LKAILALTGACG-GGKRTENPLGRCCCHSVIEQYVEELKTF	133
Wolinella	GLPKEGFSESQTRSLK--GDDLVCYCEGVTYQEIIEAIKQGATN--VREVAQATGACKSAGRCGELNPKLGRCCCAIDILEILQESG-	92
Thermoanaerobacter	-----MKVPLWFKKD-ANPKYACYCSRVTKEEIKKAVVEYGANT-MEEVINLTGAMS-NSDQKKNPDLKCCCHKIVQDAIDKGLNI	77
Chlorobium	-----MSKMMICYCSSVTEETIVSAIRRGATT--LKKIQDITGACT-VNRCKELNPSGRCCSGDILDLIERETGG	64

Fig. 7. Sequence alignment of the *A. fulgidus* CopZ domains. A, CopZ C-terminal domain sequence alignment to the *A. fulgidus* CopA N- and C-terminal MBDs and to human Atox1. Completely conserved residues are *highlighted green*; residues conserved among the *A. fulgidus* proteins are *highlighted blue*; residues conserved between CopZ-CT and CopA-NT are *highlighted yellow*; and residues conserved between CopZ-CT and CopA-CT are *highlighted red*. B, N-terminal domain sequence alignment. Sequences of homologous proteins used for the alignments were from the following species: *A. fulgidus* DSM 4304 (NP_069182.1), *Alkaliphilus metalliredigenes* QYMF (EAO82573.1), *Caldicellulosiruptor saccharolyticus* DSM 8903 (EAP42583.1), *Carboxydotherrmus hydrogenoformans*Z-2901 (YP_359666.1), *Moorella thermoacetica* ATCC 39073 (YP_429978.1), *Deinococcus geothermalis* DSM 11300 (ZP_00398040.1), *Geobacillus kaustophilus*HTA426 (YP_146024.1), *Chloroflexus aurantiacus* J-10-fl (EAO58988.1), *Thermoanaerobacter tengcongensis* MB4 (NP_623988.1), *Thiomicrospira crunogena*XCL-2 (YP_392381.1), *Thermosynechococcus elongatus* BP-1 (NP_682675.1), *Chlorobium tepidum* TLS (NP_662049.1), and *Wolinella succinogenes*DSM1740(NP_906973.1). The GenBank™ accession numbers are in *parentheses*. (Taken from Sazinsky et al. 2007)

The CopZ-NT adds an additional 130 amino acids to CopZ and is located at the N-terminus of the protein (Fig. 6). CopZ-NT has a $\beta\alpha\alpha\beta\beta\alpha$ fold and is also extremely cysteine rich (Sazinsky, LeMoine et al. 2007). CopZ-NT contains both a mononuclear zinc site and a [2Fe-2S] cluster (Fig. 8). Optical spectra done with full length CopZ, CopZ-NT and CopZ mutants indicate peaks at 340, 430, 480 nm and a shoulder at 550 nm (Sazinsky, LeMoine et al. 2007). The spectra are most similar to proteins that contain [2Fe-2S] clusters. Further supporting the presence of a [2Fe-2S] cluster, samples treated with dithionite (a reducing agent) did not show these spectral features. Due to the spectra similarities of CopZ and CopZ-NT, it appears that CopZ-CT is not involved in the assembly of the CopZ-NT metal centers. The mononuclear Zn^{2+} site was identified by flame atomic absorption and ICP atomic emission. Zn^{2+} is coordinated by a tetrahedral arrangement of Cysteines 4, 7, 38 and 43 (Sazinsky, LeMoine et al. 2007) Fig. 8B. Among all known proteins that are homologous to the N-terminus of CopZ, cysteines 4, 38 and 43 are well conserved. The mononuclear Zn^{2+} site is thought to be present for structural purposes. Since CopZ-NT has been shown to reduce Cu^{2+} the [2Fe-2S] cluster is thought to be a redox-active site (Sazinsky, LeMoine et al. 2007).

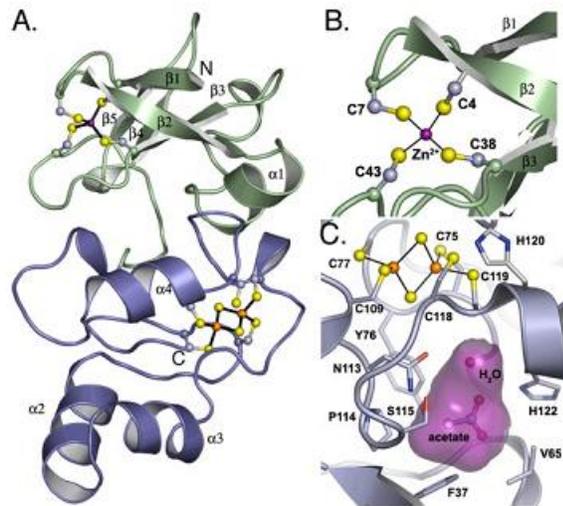


Fig. 8. Crystal structure of CopZ-NT. A, N-terminal domain of CopZ-NT is shown in *green*, and the C-terminal domain is shown in *blue*. The zinc ion is shown as a *purple sphere*, and the [2Fe-2S] cluster is shown as *yellow and orange spheres*. B, [2Fe2S] cluster. Atoms are represented as *ball and sticks* with carbon in *gray*, sulfur in *yellow*, and iron in *orange*. Acetate and water are bound in a small cavity (*magenta*) directly below the [2Fe-2S] cluster. Residues contributing to the surface of the cavity are shown as *ball-and-stick* representations. (Taken from Sazinsky et al. 2007)

1.7 Interaction of CopZ with CopA MBDS

Both CopZ domains are able to bind copper but with very different affinity values. The CopZ-CT binds copper with a K_a value of $6.6 \pm 2.2 \times 10^{14} \text{ M}^{-1}$. CopZ-NT is able to bind copper with a much lower K_a value of $4.8 \pm 1.1 \times 10^{11} \text{ M}^{-1}$ (Guerrero-Guerrero and Arguello 2008). Due to the similarities of CopZ-CT to other well-characterized metallochaperones, a higher K_a value is not surprising. The delivery of copper to cytosolic MBDS has been shown for many chaperone/ATPase pairs (Huffman and O'Halloran 2000; Walker, Tsivkovskii et al. 2002). With regard to *A. fulgidus* CopZ and CopA MBDS, our lab has shown that CopZ can deliver Cu^+ to isolated N-MBD and C-MBD of CopA (Fig. 9). The N-MBD bound copper with a K_a of $6.8 \pm 0.8 \times 10^{11} \text{ M}^{-1}$. The C-MBD completely displaced the copper from the competing BCA reagent.

Therefore, it was not possible to calculate the K_a value due to the larger value of C-MBD; nevertheless, we can however conclude that the K_a is higher than that of BCA which is $4.6 \times 10^{14} \text{ M}^{-2}$ (Guerrero-Guerrero and Arguello 2008).

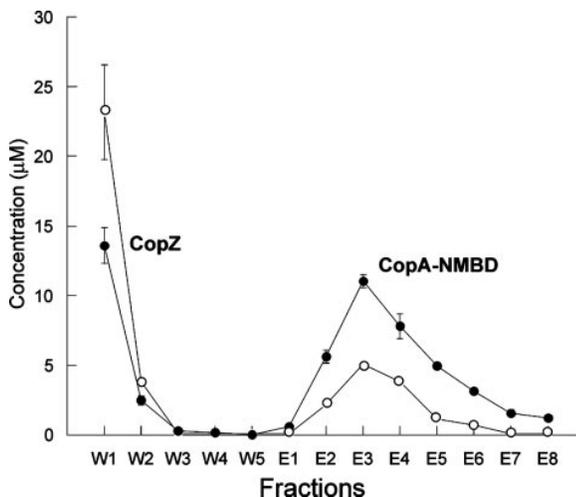


Fig. 9. Copper transfer from CopZ to the CopA N-MBD. The copper (O) and protein (●) content of the wash (W) and elution (E) fractions are shown. Peaks corresponding to specific proteins eluted from the Strep-Tactin column are identified on the figure. At the end of the experiment, 34.5% of the CopA N-MBD was loaded with copper (Taken from Sazinsky et al. 2007)

1.8 Activation of CopA by CopZ-Cu⁺

Previous studies have shown the activation of P-type ATPases by ion loaded corresponding metallochaperones (Walker, Tsivkovskii et al. 2002; Walker, Huster et al. 2004). In our laboratory experiments were carried out in order to investigate the ability of CopZ-Cu⁺ to activate CopA. As expected, CopZ-Cu⁺ was able to not only activate CopA but to do so at a much higher V_{max} of $5.5 \pm 0.2 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$. Free Cu⁺ was able to activate CopA with a V_{max} of $2.5 \pm 0.3 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$ (Guerrero-Guerrero and Arguello 2008). The $K_{1/2}$ of both CopZ-Cu⁺ and free Cu⁺ was however the same (Fig. 10).

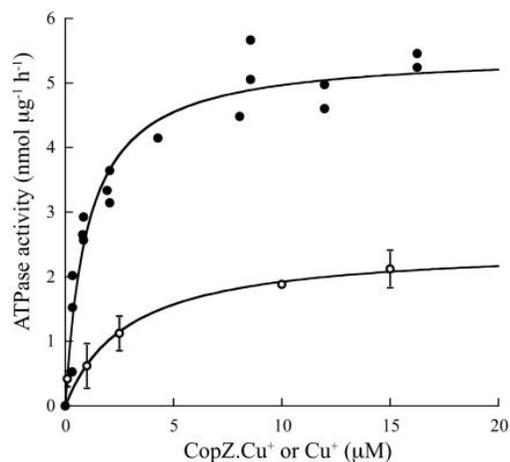


Figure. 10 CopZ-Cu⁺ activation of CopA ATPase activity. Cu⁺ was included in the assay medium as a free species (O) or bound to CopZ(●). Data were fitted by using the following parameters: free Cu⁺, $K_{1/2}=2.9\pm 1.0$ μM and $V_{max} = 2.5 \pm 0.3$ nmol μg⁻¹h⁻¹; CopZ-Cu, $K_{1/2} = 1.0\pm 0.2$ μM and $V_{max} = 5.5 \pm 0.2$ nmolμg⁻¹h⁻¹. Values obtained for CopZ-Cu⁺ were obtained in duplicate in three independent experiments. Data obtained for free Cu⁺ are the mean ± SE ($n = 3$). (Taken from González-Guerrero et al. 2008)

1.9 Interaction of CopZ with CopA

Initial models proposed that MBDs were able to translocate copper to the transmembrane metal binding site of P-type ATPases for transport. However, studies conducted in our lab have shown that the MBDs of CopA are not able to deliver copper to the transmembrane metal binding site. This was further supported by the hydrolyzation of ATP in the presence of CopZ-Cu⁺ of ΔN, C CopA, in which both MBDs have been, removed (Fig. 11) (Guerrero-Guerrero and Arguello 2008). The V_{max} of full length CopA in the presence of CopZ-Cu⁺ was 9.3 ± 0.4 nmol μg⁻¹ h⁻¹

much higher than CopA in the presence of free copper, which was $2.2 \pm 0.4 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$ (Fig. 11) (Guerrero-Guerrero and Arguello 2008).

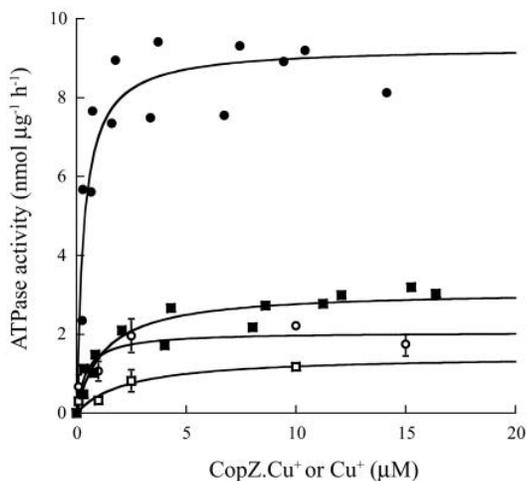


Fig. 11. CopZ-Cu⁺ activation of $\Delta\text{N,C-CopA}$ and C27,30,751,754A CopA ATPase activity. Cu⁺ was included in the assay medium either as a free species (O, □) or bound to CopZ (●, ■). Data correspond to either $\Delta\text{N,C-CopA}$ (O, ●) or C27,30,751,754A CopA (□, ■). Data were fitted by using the following parameters: $\Delta\text{N,C-CopA}$ plus free Cu⁺, $K_{1/2} = 2.4 \pm 1.2 \mu\text{M}$ and $V_{\text{max}} = 2.2 \pm 0.4 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$; $\Delta\text{N,C-CopA}$ plus CopZ-Cu, $K_{1/2} = 0.3 \pm 0.1 \mu\text{M}$ and $V_{\text{max}} = 9.3 \pm 0.4 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$; for C27,30,751,754A CopA plus free Cu, $K_{1/2} = 2.3 \pm 1.4 \mu\text{M}$ and $V_{\text{max}} = 1.2 \pm 0.3 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$; for C27,30,751,754A CopA plus CopZ-Cu, $K_{1/2} = 1.0 \pm 0.3 \mu\text{M}$ and $V_{\text{max}} = 3.1 \pm 0.2 \text{ nmol } \mu\text{g}^{-1} \text{ h}^{-1}$. Values obtained for CopZ-Cu⁺ were obtained in duplicate in three independent experiments. Data obtained for free Cu⁺ are the mean \pm SE ($n \pm 3$). (Taken from González-Guerrero et al. 2008)

The atypical N-domain of CopZ differs greatly from other metallochaperones. In fact, it is homologous to proteins found only in extremophiles and is the only such domain that is fused to a copper chaperone (Fig. 7A) (12). CopZ-NT adds an additional 130 amino acids to CopZ and has an unusually $\beta\alpha\alpha\beta\beta\alpha$ fold. CopZ-NT also contains nine cysteines, a mononuclear site and a

[2Fe-2S] cluster. Here we present evidence concerning the binding location of copper and the surprising way in which this takes place.

2. METHODS

2.1 cDNA cloning of *A. fulgidus* CopA and CopZ

All vectors and constructs used in this studies have been already reported (Sazinsky, LeMoine et al. 2007; Guerrero-Guerrero and Arguello 2008) (see table 1).

Table. 1. CopA and CopZ constructs used in this study

Construct	Tag	Reference
Δ N, C CopA*	His	{González-Guerrero, 2008 #216}
C4S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C7S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C38S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C43S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C75S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C77S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C109S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C118S CopZ	Strep	{Sazinsky, et al. 2007 #10}
C119S CopZ	Strep	{Sazinsky, et al. 2007 #10}

*CopA lacking amino acids M1-L77 and L725-S804

All CopZ constructs except for mutants C4S and C38S were transformed into *E. coil* Top10 f⁺ CP. Mutants C4S and C38S were transformed into BL21 pLysS 1240.

2.2 Protein expression in *E. coli* cells

Cells were grown at 37°C in 2xYT media (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl, 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. Cells were grown till OD₆₀₀ = 0.6, induced with 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) and allowed to grow for 3 hours. Cells were harvested by centrifugation and washed with 25 mM Tris, pH 7.0, 100 mM KCl and stored at -80°C (11).

2.3 Purification of CopZ and CopZ Mutants

CopZ and CopZ mutants were purified by affinity chromatography. Cells were resuspended with buffer W (100 mM Tris pH 8, 150 mM NaCl,) and 1 mM PMSF. Resuspended cells were disrupted by passing through a french press at 20,000 p.s.i. Cell homogenates were centrifuged at 18,000 rpm for 1 hr. Supernatants were passed through Strep-Tactin columns. Columns were washed with 6 volumes of buffer W CopZ was then eluted by the addition of buffer E (100 mM Tris pH 8, 150 mM NaCl, 2.5 mM Desthiobiotin). CopZ was concentrated using a 10 kDa centrifuge filter. Desthiobiotin was removed by the addition of 25 mM Hepes (pH 8.0) and 150 mM NaCl.

2.4 Membrane Preparation of $\Delta N,C$ CopA

Membranes were prepared as previously stated (11). Cells expressing $\Delta N,C$ CopA were harvested, resuspended in buffer A (25 mM Tris pH 7.0, 100 mM sucrose and 1 mM PMSF) and disrupted by passage through a French press at 20,000 p.s.i. Samples were then incubated with 0.02 mg/ml of DNase I and 2 mM MgCl₂ for 30 min. Broken cells were then centrifuged for 30

min at 9000 x g and supernatants spun at 163,000 x g for 1 hr. Membranes were resuspended in buffer A at a protein concentration of 2.67 $\mu\text{g}/\mu\text{l}$ and stored at -20°C (3).

2.5 Copper loading

Copper loading to apo-CopZ was carried out through incubation with either stoichiometric amounts of copper or with a 5 to 10 molar excess of a 10 mM CuSO_4 solution, 10 mM ascorbate (freshly made), 25 mM Hepes (pH 8.0), and 150 mM NaCl at room temperature for 10 min with gentle agitation. In order to ensure that samples had no carry over, as a negative control a sample was processed with no protein. As a positive control, wild type CopZ which has already been characterized for copper binding was added. The total sample volume of 100 μl was then added to a 1 ml G-25 Sephadex column in order to remove excess copper. Fractions 3 and 4 were combined and protein quantified in accordance with Bradford (7). In order to quantify bound copper, samples were treated with 10% TCA and centrifuged for 5 min at 14,000 rpm. 120 μl of supernatant was transferred to a new tube along with the addition of 10 mM ascorbate and 140 mM of bicinchoninic acid (BCA) (500 mM BCA, 260 mM NaOH and 190 mM Hepes buffer). Samples were then vortexed and allowed to incubate for 5 min at room temperature; absorbance was read at 360 nm (Brenner and Harris 1995).

2.6 ATPase Activity

5 μM CopZ was stoichiometrically loaded with copper and added to 50 mM Tris (pH 6.1 at 75°C), 3 mM MgCl_2 , 2 mM ATP, 400 mM NaCl, 2.5 mM DTT and either 10 μg or 25 μg of $\Delta\text{N,C}$ CopA. Samples were incubated at 75°C for 10 mins, and released Pi measured according

to Lanzetta et al. (Zlotnick and Gottlieb 1986). ATPases activity in the presence of apo-CopZ was subtracted.

2.7 Atomic Absorption Spectroscopy

CopZ wild type was incubated for 30 min at room temperature with a 10 molar excess of CuSO₄. Samples were passed through a G-25 Sephadex column in order to remove excess copper. Fraction 3 was collected for protein quantification by Bradford (Guerrero-Guerrero and Arguello 2008). Samples were treated with 1 ml of concentrated nitric acid (trace metal grade) at 70°C for 1 hr. Samples were allowed to cool at room temperature overnight and 250 µl of peroxide were added. Samples were brought up to a final volume of 5 ml with deionized water. In order to detect possible carryover, a 10 molar excess of CuSO₄ was processed and treated as a sample. The positive control was wild type CopZ without copper and was treated as a sample. The negative control was deionized water, concentrated nitric acid and peroxide. Metal content for all samples were measured by flame atomic absorption spectroscopy (AAS).

3. RESULTS

Previous studies done in our laboratory have concluded that CopZ-WT is able to bind two copper ions, one copper at each domain (CopZ-NT and CopZ-CT) (Sazinsky, LeMoine et al. 2007). Our goal was to identify if any of the nine cysteines located in the N-domain of CopZ was involved in the coordination of copper binding to CopZ-NT. We also wanted to know if any of these cysteines were involved in the delivery of copper to the transmembrane metal binding site of $\Delta N,C$ -CopA.

3.1 Purification and Expression of CopZ Mutants:

CopZ-WT and mutants were purified using a strep-tactin column. Mutants C4S and C38S were expressed in BL21 pLysS 1240 in order to increase protein yield. All other samples were transformed into Top 10 f' CP. Purified proteins were analyzed by SDS-PAGE to confirm proper purification. Figure 12 shows that both CopZ and CopZ mutant protein preparations are pure. This figure also shows all mutants having the same size as CopZ-WT, indicating that site directed mutagenesis did not alter the protein's molecular weight. The approximate yield for wild type CopZ, C118S, C119S, C109S and C7S was 1 mg/L of cell culture. The approximate yield for C77S, C43S, C4S and C38S was 0.3 mg/L of cell culture.

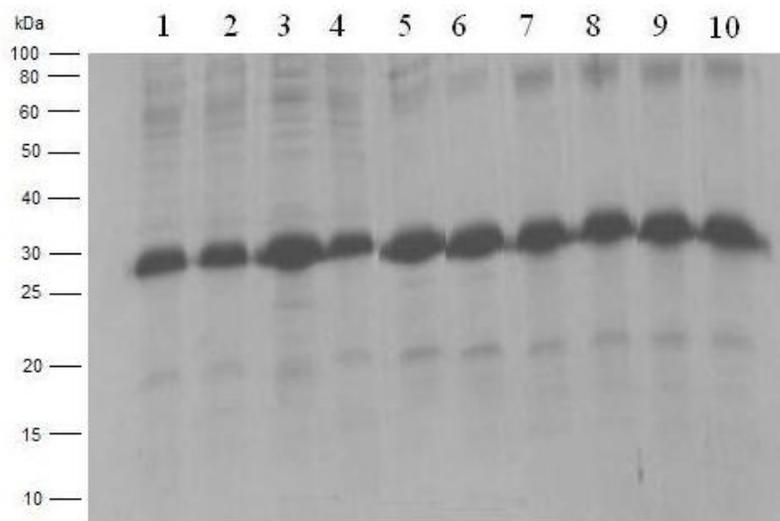


Fig. 12 Purification and Expression of CopZ Mutants: All samples were induced with IPTG and Top 10 f' CP transformed with pPR-IBA. In order to increase yield mutants from lane 2: (C4S) and lane 4: (C38S) were transformed into BL21 plysS 1240. Polyacrylamide gel stained with Coomassie brilliant blue. Lane 1: CopZ-WT, lane 2: C4S, lane 3: C7S, lane 4: C38S, lane 5: C43S, lane 6: C75S, lane 7: C77S, lane 8: C109S, lane 9: C118S and lane 10: C119S.

3.2 Copper Binding to CopZ-WT and Mutants:

In order to evaluate if individually mutated cysteines were able to affect the binding of copper in the N-domain of CopZ, copper binding experiments were carried out. Copper binding was done with apo-CopZ proteins incubated with a 10 molar excess of CuSO_4 . All samples were run through a G-25 Sephadex column in order to remove excess copper. These experiments supported previous results that indicate that CopZ-WT is able to bind two copper ions, one copper in each domain (Guerrero-Guerrero and Arguello 2008). In accordance with our hypothesis that some of the nine cysteines would be involved with the coordination of copper, for at least one of the mutant proteins we expected to see one copper bound to CopZ-CT domain and no copper bound to the N-domain. Surprisingly, all mutants bound two copper ions except for C4S and C38S. Mutants C4S and C38S demonstrated the ability to bind four copper ions

(Fig. 13). Since both C4S and C38S coordinate a Zn^{2+} atom, it was hypothesized that the displacement of Zn^{2+} may be taking place in the presence of copper (Fig. 8). It is unclear why mutants C7S and C43S were not able to bind four copper ions due to both mutants also coordinating the same Zn^{2+} ion. However, this may be due to the location of these amino acids. If both amino acids C7S and C43S are in some way sterically hindered by their location, they may not be involved in the displacement process.

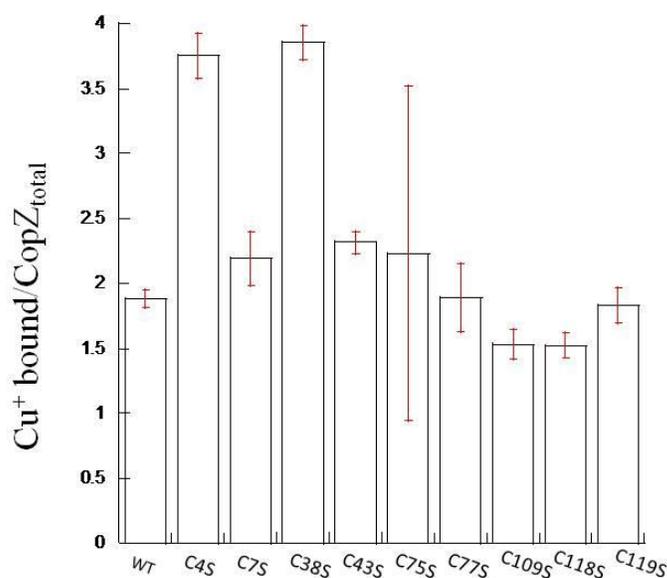


Fig. 13. Copper Binding to CopZ-WT and Mutants: Copper binding was done with apo-CopZ proteins that were incubated with a 10 molar excess of $CuSO_4$. All samples were run through a G-25 Sephadex column in order to remove excess copper. Copper determination was evaluated by the use of a BCA assay. Lane 1: CopZ-WT, lane 2: C4S, lane 3: C7S, lane 4: C38S, lane 5: C43S, lane 6: C75S, lane 7: C77S, lane 8: C109S, lane 9: C118S and lane 10: C119S.

3.3 ATPase Activity in the presence of CopZ WT and Mutants:

ATPase activation was carried out in order to evaluate if the activity of CopA would be affected by individually mutated cysteines located in the N-domain of CopZ. $\Delta N,C$ -CopA was used for all ATPase activities since it has been shown to have a threefold increase of ATPase activity in the presence of CopZ-WT versus free copper. Also the elimination of the two cytosolic domains (N and C domains) simplified our model.

Since, mutants C4S and C38S were able to bind two additional copper ions we expected to see an increase in ATPase activity. In Fig. 14, we see that both mutants more than doubled the activity seen for CopZ-WT. However, we also see that mutant C118S has an equal amount of ATPase activity compared to mutants C4S and C38S. The reason behind this high ATPase activity of C118S is unclear.

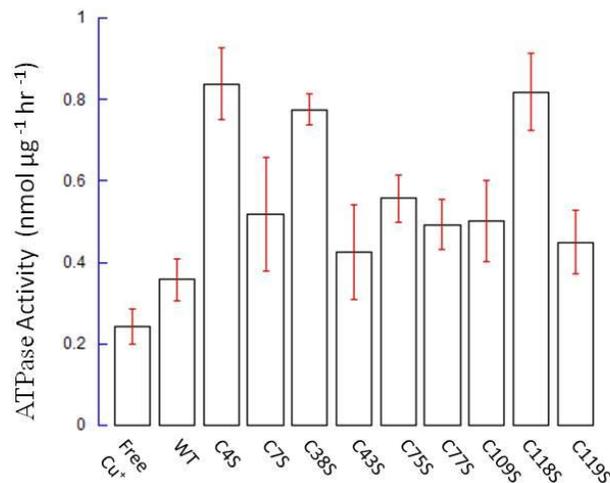


Fig. 14. ATPase Activity of Membrane $\Delta N,C$ -CopA in the presence of CopZ-WT or Mutants: Activation of membrane $\Delta N,C$ -CopA by 5 μM concentrations of either free copper or CopZ-Cu⁺ proteins. ATPase Activity took

place in medium containing 50 mM Tris (pH 6.1 at 75°C), 3 mM MgCl₂, 2 mM ATP, 400 mM NaCl, 2.5 mM DTT and 10 µg of ΔN,C-CopA.

Cysteine has been shown to increase the turnover rate of ΔN,C-CopA by allosteric interactions (Yang, Mandal et al. 2007). Thus, ATPase activities for all samples were measured in the absence and in the presence of cysteine (Fig. 14 and 15 respectively). Due to the combination of CopZ and cysteine we expected to see a much large ATPase activity overall. However, this was not the case. Though the addition of cysteine allowed for much less variability there was no noticeable increase in ATPase activity compared to Fig. 14. Also the unexpected increase in ATPase activity that was noticed in Fig. 14 for mutant C118S was not present. Unfortunately, we were not able to measure mutants C4S and C38S in the presence of cysteine due to the consistent low yield obtained in multiple purifications. However, we suspect the reproducibility of the high ATPase activity value in the presence of cysteine.

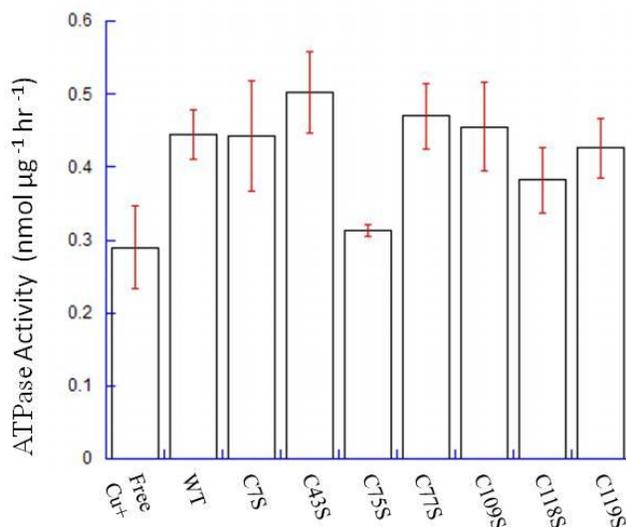


Fig. 15. ATPase Activity of Membrane Δ N,C-CopA in the presence of Cysteine and CopZ-WT or Mutants: Activation of membrane Δ N,C-CopA by 5 μ M concentrations of either free copper or CopZ-Cu⁺ proteins. ATPase Activity took place in medium containing 50 mM Tris (pH 6.1 at 75°C), 3 mM MgCl₂, 3 mM ATP, 400 mM NaCl, 2.5 mM DTT, 10 mM cysteine and 25 μ g of membrane Δ N,C-CopA.

3.4 Atomic Absorption spectroscopy (AAS):

The copper binding assay indicated that mutants C4S and C38S are able to bind four copper ions. Since these Cys coordinate a mononuclear zinc site located in the N-Domain of CopZ, we hypothesized that the coordination of Cu⁺ may take place by the displacement of Zn²⁺ (Fig. 8B). In order to explore this hypothesis CopZ-WT was incubated in the presence and absence of a 10 molar excess of Cu⁺. The presence of Zn²⁺ was evaluated by atomic absorption spectroscopy (AAS). As shown in Fig. 17, in the absence of Cu⁺, Zn²⁺ is present at 0.672 ± 0.377 ppb. However, in the presence of Cu⁺, zinc read 0.008 ± 0.014 ppb. These results indicate the complete removal of Zn²⁺ from its binding pocket (Fig.16). Although, there are many proteins

that function by displacing one metal for another, this is unprecedented for a metallochaperone. However, due to the uniqueness of CopZ-NT it is not surprising. To date no other metallochaperone has been indentified that contains an additional 130 amino acids, a [2Fe-2S] cluster, and a mononuclear zinc site. The fusion of these two domains CopZ-NT and CopZ-CT may completely be by evolutionary change. Nevertheless, due to the favorable outcome of being able to sequester more Cu^+ and an internal reducing agent, *A. fulgidus* has benefited.

Experimental Conditions	Zinc Presences ppb
In the absence of Cu^+	0.672 ± 0.377
In the presence of Cu^+	0.008 ± 0.014

Fig. 16. Flame atomic absorption spectroscopy (AAS) was used to determine the presences of zinc in CopZ-WT in the presences and absence of a 10 molar excess of CuSO_4 .

4. DISCUSSION

Previous experiments done in our laboratory have shown that CopZ-WT is able to bind two copper ions, one copper at each domain (N and C domains). CopZ-CT is homologous to well characterized metallochaperones. However, CopZ-NT is homologous to proteins found only in extremophiles and is the only such protein that is fused to a copper chaperone (Fig. 7A) (12). The N-domain of CopZ is extremely cysteine rich (Fig. 8). The nine cysteines located in CopZ-NT have all been individually mutated in order to determine if any of the cysteines play a role in copper binding and in ATPase activity.

Copper binding experiments revealed that two of the nine mutants affect copper binding (Fig. 13). Mutants C4S and C38S indicated a copper binding capacity of four. These results were extremely unexpected. We were expecting to see a copper binding of one to the CopZ-CT domain and no copper bound to the cysteine or cysteines that contribute in the CopZ-NT domain. However, both mutants C4S and C38S are involved in the coordination of a zinc mononuclear site located in the N-Domain of CopZ (Fig. 8B). Therefore, we hypothesized that the coordination of copper may take place by the displacement of zinc. It is unclear why mutants C7S and C43S do not also display the ability to bind two additional copper ions since both mutants also coordinate to the same zinc mononuclear site. However, if both C7 and C43 are in some way sterically hindered by their location, they may not be involved in the displacement process.

In order to explore Zn^{2+} displacement in mutants C4S and C7S, atomic absorption was carried out. CopZ-WT was incubated in the absence and presence of a 10 molar excess of copper. Excess copper was removed by a G-25 Sephadex column and proteins were digested by

nitric acid. Metal contents were read by atomic absorption. The experimental results indicated that without the addition of copper, the amount of zinc present was 0.672 ± 0.377 ppb (Fig. 16). However, after CopZ-WT was allowed to incubate with copper, zinc was measured at a 0.008 ± 0.014 ppb. These results demonstrated the complete displacement of zinc by copper. Although, there are many proteins that function in this manner, CopZ is the only known metallochaperone that obtains the metal of interest by the displacement of another. However, due to the nontraditional characteristics of CopZ-NT this is not surprising. It is possible that during the evolution of *A. fulgidus*, these two domains (CopZ-NT and CopZ-CT) became fused together by chance and due to the increased binding capacity and the internal reducing ability stayed joined. It was not possible to repeat the atomic absorption with mutants C4S and C38S due to the low yield obtained after multiple protein purifications.

Experimental results for ATPase activity was carried out in order to evaluate if the activity of $\Delta N,C$ -CopA would be affected by individually mutated cysteines. Cysteine has been shown to increase the turnover rate of P-type ATPases through allosteric interaction (Yang, Mandal et al. 2007). Therefore, ATPase activities were carried out in the absence and presence of cysteine (Fig. 14 and 15 respectively). Because the activity of $\Delta N,C$ -CopA has been shown to increase in the presences of CopZ-WT compared to free copper and to simplify our model $\Delta N,C$ -CopA was used for all ATPase activities.

The expected increase in the activity of $\Delta N,C$ -CopA in the presences of CopZ-WT compared to free copper was observed for both ATPase activities (Fig.14,15). However, the difference between CopZ-WT and free copper was not as significant as expected. Also in Fig. 15 mutant C75S and free copper had very similar activities (0.313 nmol/ μ g/h and 0.290 nmol/ μ g/h respectively). However, experiments done showing an increase in the activity of $\Delta N,C$ -CopA in

the presence CopZ-WT was done with purified $\Delta N,C$ -CopA and not membrane $\Delta N,C$ -CopA therefore the experimental conditions may not be favorable for this interaction.

We expected to see an increase in ATPases activity for mutants C4S and C38S due to the addition of two copper ions bound to CopZ-NT domain. As expected in Fig. 15 we did see a noticeable increase in activity for both mutants C4S and C38S. However, the same increase in activity was seen with mutant C118S. It is unclear why mutant C118S is displaying such a large increase in ATPase activity. However, for ATPase activities done with the addition of cysteine we see that mutant C118S is displaying the same activity values as all other mutants (Fig. 15).

Unfortunately, we were not able to measure mutants C4S and C38S in the presence of cysteine due to the consistent low yield obtained in multiple purifications. However, we suspect the reproducibility of a high ATPase activity value in the presence of cysteine for both C4S and C38S.

We had anticipated seeing a larger amount of activity overall for ATPase activities done with the addition of cysteine however this was not the case. Although, the addition of cysteine allowed for more consistent results there was still no overall increase seen when comparing Fig 14 and 15. Also the amount of membrane $\Delta N,C$ -CopA added in Fig 14 and 15 were 10 μ g and 25 μ g respectively. It is unclear why even with a large amount of $\Delta N,C$ -CopA we did not obtain a much larger value for the ATPase activity of membrane $\Delta N,C$ -CopA in the presence of cysteine.

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