

JMA MQP 0830

**Mechanisms of Cu⁺ Delivery From
Archaeoglobus fulgidus CopZ to CopA**

A Major Qualifying Report:

submitted to the Faculty

of

Worcester Polytechnic Institute

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

Brad M. Kaufman

Date: December 17, 2008

Approved:

Professor José M. Argüello
Biochemistry
WPI Project Advisor

Abstract

Cu⁺-ATPases are involved in efflux of copper from the cytoplasm. Under physiological conditions these ATPases receive the metal from a specific metallochaperone. We hypothesize that the specificity of the interaction is provided by electrostatic interactions between the transporter and its chaperone. If there are charged or polar amino acids that lie on the rim of the chaperone around the copper binding residues, they could potentially interact with the transporter during copper delivery. To test this hypothesis, I used a model system with a Cu⁺ chaperone (*Archaeoglobus fulgidus* CopZ) and analyzed the effects on the ATPase activity of its respective transporter (CopA). I created mutants in CopZ that replaced charged residues (Asp, Ser, Thr, and Tyr) and measured the effect on the activation of CopA by CopZ. If the residues were involved in copper delivery, there would be an increase in the K_{1/2} or a decrease in the V_{max} of the ATPases with the mutant CopZ molecules. There appears to be no significant effect on activity when the Asp is mutated to either an Ala or a Lys.

Table of Contents

Signature Page	1
Abstract	2
Table of Contents	3
Acknowledgements	4
Introduction	5
Materials and Methods	8
Results	14
Discussion	20
References	22

Acknowledgements

I would like to thank Professor José Argüello for allowing me to learn how to work in a scientific lab and providing me with invaluable knowledge. I would also like to thank Manuel González-Guerrero for answering all of my questions, even if it was with another question.

Introduction

In small amounts, heavy metals serve important roles in living organisms such as respiration, collagen maturation, and pigmentation (1). In contrast, excess free metal in the cell leads to free radicals and irreversible binding to proteins (2). For this reason, mechanisms to control cellular concentrations of metal are necessary. These mechanisms include chelating agents, chaperones, and transport (efflux and influx) proteins which have a high affinity for the metals (1).

P-type ATPases are a family of membrane proteins characterized by the E1/E2 Albers-Post catalytic cycle (Fig. 1, (3)). In the cytoplasmic side of the membrane, ATP binds the ATP binding domain (ATP-BD) and the metal binds to the transmembrane metal binding sites (TM-MBS) with high affinity in the E1 conformation (step 4). After ATP phosphorylation, the enzyme changes conformation and the metal is not accessible from the cytoplasm (step 1). Next, the metal leaves the enzyme which is in the E2P conformation (step 2). The P_i is released from the enzyme to yield the E2 conformation (step 3). The enzyme then returns to the E1 conformation in the presence of ATP and Cu^+ (3).

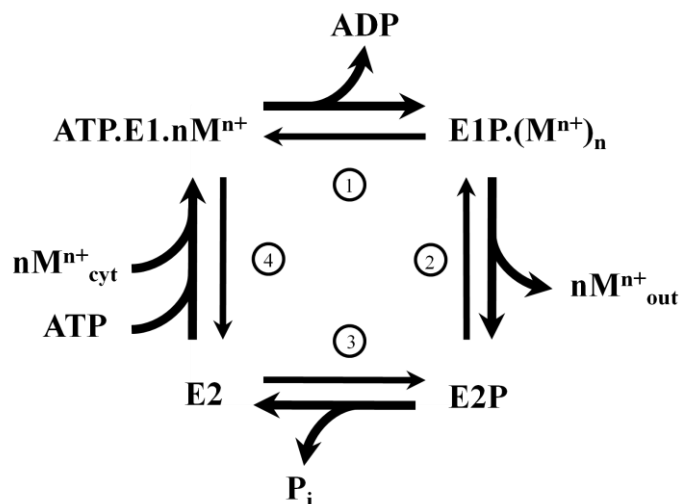


Figure 1. P_{1B}-ATPases catalytic cycle. E₁, E₁P, E₂, and E₂P are the four main conformations that the enzyme can have. Mⁿ⁺ is the metal transported by the enzyme and n is the stoichiometry of the metal being transported. Mⁿ⁺_{cyt} and Mⁿ⁺_{out} are the cytoplasmic or extracellular locations of the metal (3).

P_{1B}-type ATPases are a subfamily of P-type ATPases that transport heavy metals, including Cu⁺, Cu²⁺, Zn²⁺, and Co²⁺ across the membrane out of the cytoplasm (4). These proteins are found in most organisms (4). Characteristics of P_{1B}-ATPases include 8 or 6 transmembrane segments (TMs), a regulatory cytoplasmic metal binding domain (MBD), an ATP binding domain, and a signature metal-binding sequence in the TMs (3).

CopA has 8 TMs with cytoplasmic loops. Within the TMs, there are two TM-MBS. These sites consist of conserved residues that coordinate and move the metal across the membrane (5,6). In the sixth TM, the conserved sequence is CPC. In the seventh TM, Asn and Tyr are conserved. Met and Ser are conserved within the eighth TM (Fig. 2, (4)).

Between TM 6 and 7, there is a large cytoplasmic loop that has a conserved DKTGT sequence. This loop is the ATP-BD and consists of the nucleotide binding (N) and the phosphorylation (P) domains. The ATP binding domain has a high affinity for ATP, but is much lower in the E2 conformation. The DKTGT is located in the P- domain, where the Asp becomes phosphorylated when the ATP is used. There is a smaller loop between helix 4 and 5 which is the actuator (A) domain (3).

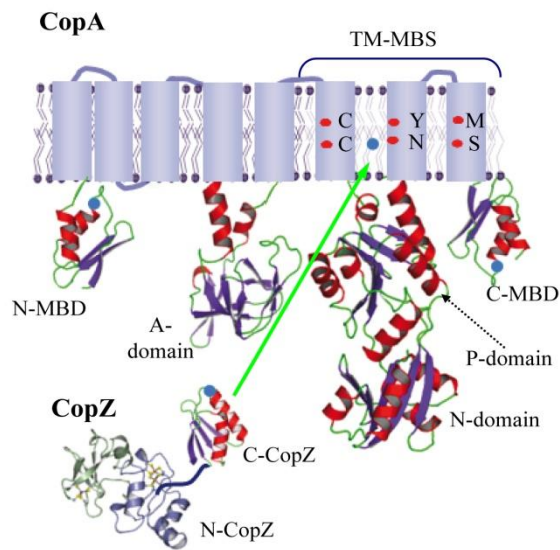


Figure 2. Diagram of CopA, showing the 8 TMs. Below the TMs, crystal structures obtained from the Protein Data Bank (PDB) are shown of the soluble A-domain (PDB ID code 2HC8), and the ATP-BD (PDB ID code 2B8E). The N- and C- terminal metal binding domain (N-, C- MBD) crystal structures are homology models based on the fourth N-MBD Menkes protein (PDB ID code 1AW0) and *Bacillus subtilis* CopZ (PDB ID code 1K0V). The conserved amino acids in TM 6 – 8 that make up the TM-MBS are also shown. The CopZ protein shown includes the N-domain structure (PDB ID code 2HU9) and a C-domain homology model based on *Enterococcus hirae* CopZ (PDB ID code 1CPZ). The green arrow indicates the delivery path WT CopZ takes (7).

CopA has cytoplasmic N- and C-terminal MBDs. These have a conserved CXXC metal binding sequence (8). A proposed mechanism is that in the absence of copper, the metal binding domains bind to the ATP-BD and prevent copper transport. When copper binds the MBDs, it detaches and allows the enzyme to transport copper (3).

Humans have two $P_{1B}\text{-Cu}^+\text{-ATPases}$ that are important in transporting copper out of the cell, ATP7A and ATP7B (9). Mutations in these proteins leads to Menkes and Wilson's diseases (9). An analog of these proteins is *Archaeoglobus fulgidus* CopA which transports Cu^+ (11).

Copper cannot be free inside the cell because it is highly toxic (7). Therefore, most of the copper is complexed in a metallochaperone. CopZ is a Cu^+ chaperone in *A. fulgidus* (12) which delivers copper to CopA *in vitro* (7). CopZ is 23 kDa (29.8 kDa with a His tag) and has two domains, the N- and C- domains. It binds 2 copper ions, 1 in each domain. The N-domain is a unique domain compared to other Cu^+ chaperones. It is 14 kDa large and has no known function, however, it can reduce Cu(II) to Cu(I). There is a [2Fe-2S] cluster and a mononuclear zinc site (12). The C-domain is 9 kDa and has orthologues in many organisms (13). This domain contains two cysteines at amino acids 149 and 152 which coordinate the copper cation and has the characteristic CXXC metal binding sequence (13).

CopZ can deliver Cu^+ to the N- and C- MBD (6). However, the MBDs are not able to activate CopA. CopA without the N- and C- MBDs has the same V_{\max} as WT CopA which supports the hypothesis of the domains having a regulatory role.

The objectives of this project were to find and mutate residues in CopZ that potentially participate in the interaction of CopZ with CopA when delivering copper and to observe the effect of these mutants on the ATPase activity of CopA. The mutations may change the affinity of CopZ for CopA, so the effects being looked at are changes in the V_{\max} and the $K_{1/2}$.

Materials and Methods

Homology Modeling of Ct-CopZ: A suitable template for homology modeling of Ct-CopZ, the HMA domain of the copper chaperone for *Homo sapiens* superoxide dismutase (2CRL), was identified by performing a Fasta search (14) in the Protein Data Bank repository of protein structures. Ct-CopZ structures were modeled using SWISSMODEL (15), (16) and were visualized with PyMol (Delano Scientific). Electrostatic models were obtained using the APBS plugin for PyMol (17).

CopZ and $\Delta N,C$ -CopA Cloning: The *A. fulgidus* CopA construct was previously made by cloning the CopA cDNA into a pBAD/Topo vector (11) and transformed into *E. coli* Top10 cells (Invitrogen). The $\Delta N,C$ -CopA construct (CopA with the N- and C-metal binding domains absent) was made by carrying out a Polymerase Chain Reaction (PCR) with primers at Gly⁸⁰ and Gly⁷³⁶ using WT CopA as a template (7). To create the CopZ mutants, a pBAD/Topo vector (Invitrogen) containing CopZ cDNA was used as a template. Site directed mutagenesis was performed using PCR with primers (Sigma Genosys) designed to introduce mutations and a novel restriction site (Table 1). These mutations were T148A, S154A, D173A, D173K, and Y197A. To create the T148A, D173A, and Y197A mutants, the respective mutant primers (both 5' to 3' and 3' to 5') were used to amplify the full CopZ/pBAD vector. PCRs were performed in a 50 μ L reaction containing 5 mM of each primer, 5 mM dNTP, 1x red Taq buffer (Sigma), 1 μ L red Taq polymerase (Sigma), and 20 ng template DNA in water. Regular Taq polymerase and Taq buffer (Sigma) were used if screening. The temperature conditions for all PCRs were:

1. 95 °C 5 min
2. 95 °C 30 sec
3. 55 °C 30 sec
4. 72 °C 6 min
5. 35 cycles to step 2
6. 72 °C 7 min
7. 4 °C 24 h

Methylated bacterial template DNA was digested by incubating 1 µl DpnI with 35 µl of PCR product at 37 °C for 1 h and was then gel purified (Qiagen). The purified DNA was then transformed as described below. The D173K mutant was made using the megaprimer method (18). This involved 2 PCR steps. In the first step, the primers used were 5' D173K and 3' CopZ. This created the 3' megaprimer, which was gel purified (Qiagen). Next, the 3' megaprimer was used with 5' CopZ primer to create the full length CopZ sequence. The amplicon DNA was A-tailed by adding 25 µl DNA to a buffer containing 1X Taq polymerase buffer, 0.3 µM dATP, and 1 µl Taq polymerase (Sigma) and incubated at 72 °C for 15 min. The A-tailed DNA was gel purified and then ligated into the pBAD/Topo vector (Invitrogen). This vector encodes an ampicillin resistance gene, is under the control of an arabinose inducible promoter, and introduces a (His)₆ tag at the C-terminus of CopZ. CopZ cDNA in the pBAD/Topo vector was gel purified (Qiagen) to prevent any contaminant (not CopZ) DNA from entering the bacteria. The vector was transformed into Top10 Competent *E. coli* cells (Invitrogen). Only the bacteria transformed by the pBAD/Topo vector grew on a 2x YT + ampicillin (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 100 µg/ml ampicillin, and 2% agar) plate. The plates were grown

overnight at 37 °C. Colonies were screened by taking them with a pipette tip and agitating the bacteria in the PCR mix. Primers used in the PCR were 5' CopZ and 3' pBAD (Table 1) to check for correct orientation in the vector. A restriction digestion was performed with 8 µl of amplicon DNA from the PCR, 1 µl unique restriction enzyme (Table 1), and 1 µl appropriate buffer. The solution was incubated at 37 °C for 1 h. An agarose gel was run with the undigested amplicon DNA next to the digested DNA. Full sequence confirmation was obtained by sending vector DNA containing mutant CopZ to be sequenced by MacroGen.

Table 1. Sequences of primers used to introduce mutations in CopZ DNA. The underlined letters are the changed nucleotides. The bold letters are the restriction site.

Name	5' to 3' Sequence	Restriction Enzyme
T148A	GACGGTCTC <u>G</u> CATGCATGGGG	SphI
S154A	GGGGTGTGTTG <u>C</u> A <u>G</u> GCTGTAAAG	PvuII
D173A	ATTGGGCTGG <u>C</u> <u>C</u> GGGCTGTT	SmaI
D173K	ATTGGGCTT <u>A</u> <u>A</u> GAGGGCTGTT	AflII
Y197A	GGAGCAGG <u>C</u> <u>G</u> CCTCTGCACGG	NarI
5' CopZ	ATGATGCGATGCCCAGAATG	N/A
3' CopZ	TCTCTTTCAAGCCGTGCAGA	N/A
3' pBAD	GATTTAATCTGTATCAGGCT	N/A

Protein Expression in E. coli: The bacteria was grown in 2x YT media (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl) with 100 µg/ml ampicillin at 37 °C until an OD₆₀₀=0.6, when they were induced with 0.02% L-arabinose. The cells were harvested 3 h after induction, washed in buffer containing 25 mM Tris-HCl (pH 7.0) and 100 mM KCl, and stored at -80 °C.

Protein Purification: Cells containing overexpressed protein were resuspended in buffer containing 25 mM Tris, pH 7.0, 100 mM sucrose, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). They were lysed by passing through a French Press at 20,000 p.s.i. three times. The

lysate was centrifuged at 9,000 rpm for 30 min. Supernatant of $\Delta N,C$ -CopA preparations was centrifuged at 33,000 rpm for 1 h. The pellet was resuspended in buffer B (25 mM Tris, pH 8.0, 100 mM sucrose, 500 mM NaCl, 1 mM PMSF) and homogenized using a hand homogenizer. The homogenized membranes were solubilized by diluting to 3 mg/ml protein in buffer B and adding 0.75% n-Dodecyl- β -maltoside (DDM) dropwise. This solution was stirred for 1 h at 4°C. Unsolubilized membrane was removed by centrifuging for 1 h at 33,000 rpm. The supernatant was incubated with Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin for 1 h at 4°C. The solution was passed through a column and washed with 10 ml buffer B with 5 mM imidazole. The resin was washed a second time with buffer B with 20 mM imidazole and eluted with 10 ml buffer B with 150 mM imidazole. The elution was concentrated using a 30 kDa centrifuge filter. Imidazole was removed by washing with buffer containing 25 mM Tris, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 1 mM dithiothreitol (DTT). When purifying CopZ, the steps were the same as above until the supernatant from the 30 min, 9,000 rpm centrifuge was passed through the Ni-NTA column as above. CopZ protein was concentrated using a 10 kDa centrifuge filter. Imidazole was removed using the same buffer as above without DDM. Protein was stored at -80°C after adding 10% glycerol. Protein concentrations were determined by the method described by Bradford (19).

Dot Blot to Confirm Expression: Aliquots (2 ml) of bacteria were collected and pelleted before induction and 3 h post induction to check expression levels of the mutants. Pellets were resuspended in 50 μ l water and lysed using a sonicator at 40 watts for 30 sec. SDS was added to a concentration of 0.1% and the samples were boiled for 5 min. The protein in the sample was quantified. Cell lysate was pipetted onto a nitrocellulose membrane; 5 μ g uninduced, 5 and 10

μg induced, 10 μg BSA (negative control), and 5 μg CopA with a His tag as a positive control. The membrane was allowed to dry. It was then rinsed with buffer 229 (50 mM Tris pH 7.4 and 0.2 M NaCl), then blocked with buffer 229 with 5% nonfat dry milk (blocking solution) for 20 min with horizontal rocking. Blocking solution and a 1:1000 dilution of Anti-His primary antibody (Santacruz) was added and rocked overnight at 4 °C. The antibody was poured off and washed with buffer 229 with 0.05% Tween 20 twice, then twice with 5 min rocking. There was one last wash with just buffer 229 for 5 min, then a 1:1000 dilution of the secondary antibody (Amersham) in blocking solution was rocked for 1 h at room temperature. The antibody was poured off and the membrane was washed as stated above. The membrane was exposed to ECL solution (Thermo Scientific) and visualized using Bio-Rad Gel/Chemi Doc.

CopZ Copper Loading: A copper loading/binding experiment was performed to determine if CopZ mutants could be loaded stoichiometrically or in 5x excess of copper. Standards were made with CuSO_4 . There were three reactions: copper with no protein, protein with stoichiometric copper, and protein with 5x copper. The reaction volume was 100 μl and contained 10 μM CopZ with 5 mM ascorbate (freshly prepared) and CuSO_4 (10 or 50 μM). The negative control had 50 μM CuSO_4 with no protein. Reactions were rocked for 10 min at room temperature. The loaded protein was passed through 1 ml Sephadex G-25 and eluted with 100 μl of water in each of 4 elution fractions. Protein in reactions 3 and 4 were quantified according to Bradford (19). Approximately 0.25 nmol protein was added to a new tube. The volume was completed to 70 μl with water. TCA (10 μl of 60%) was added and the samples were vortexed. Samples were centrifuged at max speed for 5 min and 75 μl of the supernatant was transferred to a new tube. Ascorbate (5 μl of 5 mM) and 20 μl of bicinchoninic acid (BCA) solution (500 μM

BCA, 260 mM NaOH, and 190 mM HEPES acid) were added next and vortexed. The absorption was read at 360 nm (20).

ATPase Assays: Purified CopZ was loaded with a 2:1 molar ratio of CuSO₄ and 10 mM ascorbate in water for 10 min at room temperature. ATPase assays were performed in a solution containing 50 mM Tris (pH 7.5 at RT), 3 mM MgCl₂, 3 mM ATP, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 2.5 mM DTT, 2.5 μg purified ΔN,C-CopA, and various concentrations of CopZ. A control with Cu-free CopZ was used as a blank for each concentration of CopZ. ATPase activity was measured after 10 min incubation at 75°C. Released P_i was determined according to Lanzetta et al. (21). ATPase activity of CopZ without metal was subtracted from measured values. Curves of ATPase activity vs. donor·Cu⁺ (CopZ) concentrations were fit to $v = V_{\max}[\text{donor}\cdot\text{Cu}^+]/([\text{donor}\cdot\text{Cu}^+] + K_{1/2})$. The standard errors for V_{max} and K_{1/2} are asymptotic standard errors reported by the fitting software KaleidaGraph (Synergy).

Results

The goal of this project was to study interactions CopZ has with CopA during the process of delivering copper. To accomplish this goal, mutants of *A. fulgidus* CopZ were made and then the effect of the mutants on the ATPase activity of *A. fulgidus* CopA was analyzed.

Homology modeling: The homology modeling of CopZ showed a similar structure to the model of the HMA domain of the copper chaperone for *H. sapiens* superoxide dismutase (Fig. 3). The copper coordinating cysteines as well as the alpha helices and beta sheets were in comparable locations. An electrostatic model of Ct-CopZ was made to determine the locations of polar residues with respect to the conserved cysteine residues (Fig. 4). The polar residues that surrounded the cysteines were T148, S154, D173, and Y197.

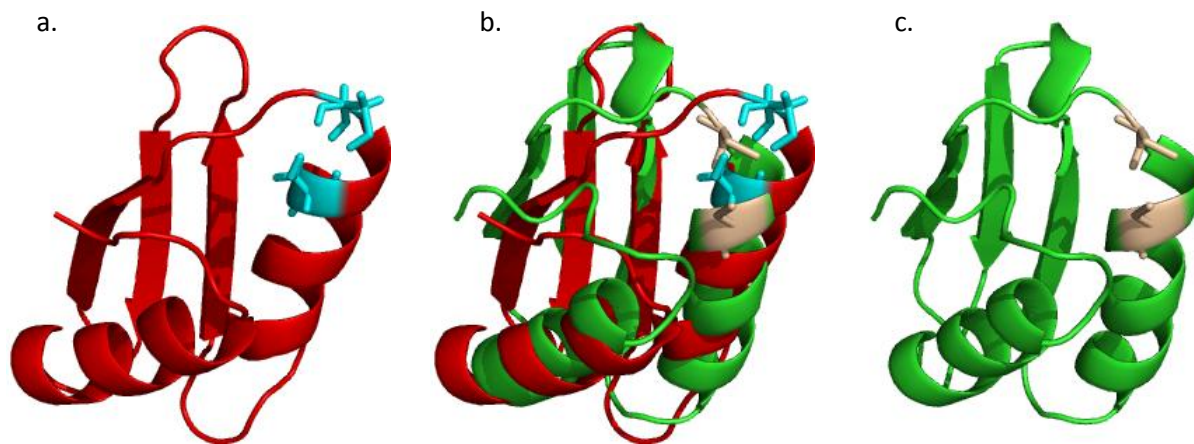


Figure 3. Homology modeling of Ct-CopZ and a crystallized homologue. Figure a. is the HMA domain of the copper chaperone for *H. sapiens* superoxide dismutase, Figure c. is *A. fulgidus* Ct-CopZ and Figure b. is an alignment of the two proteins. The copper coordinating cysteines are in blue (*H. sapiens*) and tan (*A. fulgidus*).

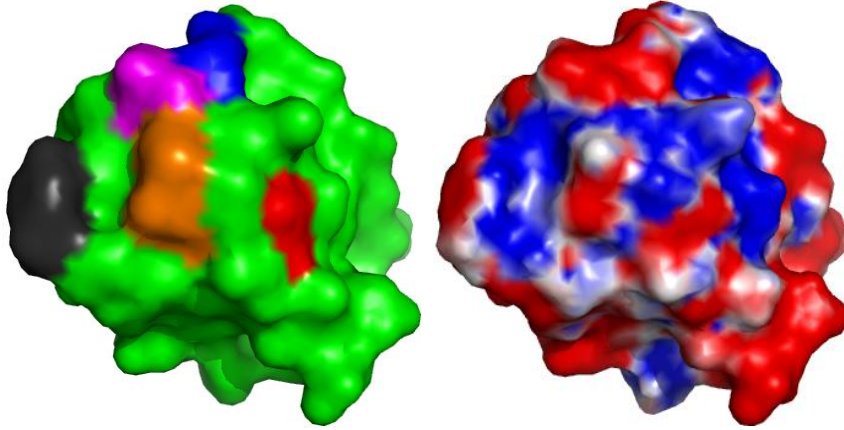


Figure 4. Electrostatic model of *A. fulgidus* Ct-CopZ. Figure a. shows the residues that potentially interact with CopA. D173, S154, T148, and Y197 are blue, red, magenta, and black, respectively. The coordinating cysteines are in orange. Figure b. shows an electrostatic model of Ct-CopZ, where red is negatively charged and blue is positively charged. The points on a. correspond to the points on b.

CopZ Mutant Cloning: The mutant CopZ PCR product was confirmed by digesting with a unique restriction enzyme and running on an agarose gel (Fig. 5). Only 4 of the 5 mutants were obtained (T148A, D173A, D173K, and Y197A). Both the full plasmid amplification and the megaprimer PCR techniques were tried multiple times to obtain S154A with no success. Macrogen sequencing verified that only the intended mutations were in the CopZ mutant DNA (Fig. 6).

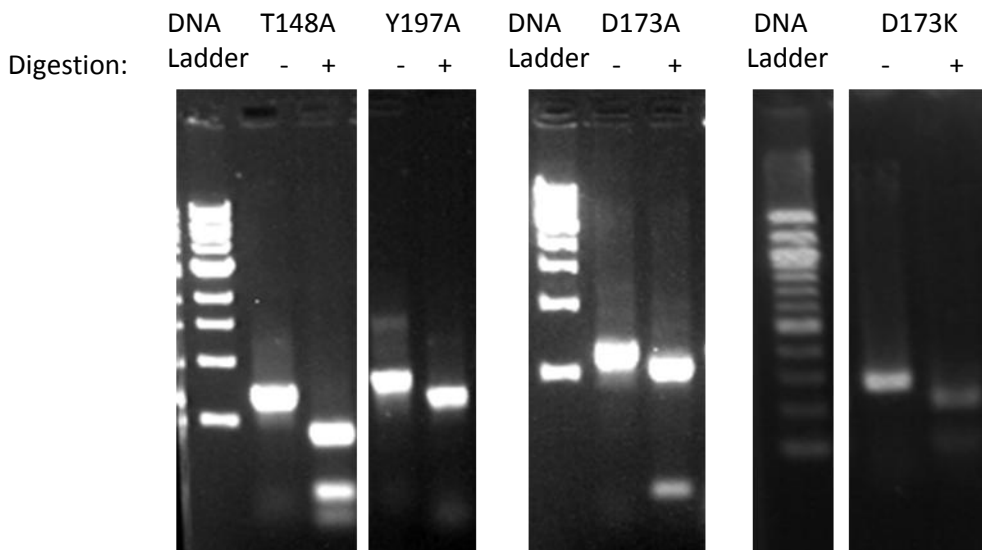


Figure 5. Digestion confirmation of T148A, Y197A, D173A, and D173K. T148A DNA was digested with SphI. Y197A DNA was digested with NarI. D173A DNA was digested with SmaI. D173K DNA was digested with AflIII.

a.

```

WT CopZ      CTCACATGCATGGGGTGTGTTAGCGCTGTAAAGGCGGCTCTTGAAGAGGCCGGAGCGAAT 60
D173A CopZ   CTCACATGCATGGGGTGTGTTAGCGCTGTAAAGGCGGCTCTTGAAGAGGCCGGAGCGAAT 60
D173K CopZ   CTCACATGCATGGGGTGTGTTAGCGCTGTAAAGGCGGCTCTTGAAGAGGCCGGAGCGAAT 60
S154A CopZ   CTCACATGCATGGGGTGTGTTGCAAGCTGTAAAGGCGGCTCTTGAAGAGGCCGGAGCGAAT 60
T148A CopZ   CTCGCATGCATGGGGTGTGTTAGCGCTGTAAAGGCGGCTCTTGAAGAGGCCGGAGCGAAT 60
Y197A CopZ   CTCACATGCATGGGGTGTGTTAGCGCTGTAAAGGCGGCTCTTGAAGAGGCCGGAGCGAAT 60

WT CopZ      GTTGTGAAATTGGGCTGGACAGGGCTGTTGTTGAGGTTGACGAGGAAGCGGAGCTGCAG 120
D173A CopZ   GTTGTGAAATTGGGCTGGCCGGGCTGTTGTTGAGGTTGACGAGGAAGCGGAGCTGCAG 120
D173K CopZ   GTTGTGAAATTGGGCTTTAAGAGGGCTGTTGTTGAGGTTGACGAGGAAGCGGAGCTGCAG 120
S154A CopZ   GTTGTGAAATTGGGCTGGACAGGGCTGTTGTTGAGGTTGACGAGGAAGCGGAGCTGCAG 120
T148A CopZ   GTTGTGAAATTGGGCTGGACAGGGCTGTTGTTGAGGTTGACGAGGAAGCGGAGCTGCAG 120
Y197A CopZ   GTTGTGAAATTGGGCTGGACAGGGCTGTTGTTGAGGTTGACGAGGAAGCGGAGCTGCAG 120

WT CopZ      AAACTCGTTGAGGCTGTTGAGGGAGCAGGTTACTC 155
D173A CopZ   AAACTCGTTGAGGCTGTTGAGGGAGCAGGTTACTC 155
D173K CopZ   AAACTCGTTGAGGCTGTTGAGGGAGCAGGTTACTC 155
S154A CopZ   AAACTCGTTGAGGCTGTTGAGGGAGCAGGTTACTC 155
T148A CopZ   AAACTCGTTGAGGCTGTTGAGGGAGCAGGTTACTC 155
Y197A CopZ   AAACTCGTTGAGGCTGTTGAGGGAGCAGGCCGCCTC 155

```

b.

```

WT CopZ      LTCMGCVSAVKAALEEAGANVVEIGLDRAVVEVDEEAELQKLVEAVEGAGY 51
D173A CopZ   LTCMGCVSAVKAALEEAGANVVEIGLRAVVEVDEEAELQKLVEAVEGAGY 51
D173K CopZ   LTCMGCVSAVKAALEEAGANVVEIGLRAVVEVDEEAELQKLVEAVEGAGY 51
S154A CopZ   LTCMGCVAVKAALEEAGANVVEIGLDRAVVEVDEEAELQKLVEAVEGAGY 51
T148A CopZ   LAACMGCVSAVKAALEEAGANVVEIGLDRAVVEVDEEAELQKLVEAVEGAGY 51
Y197A CopZ   LTCMGCVSAVKAALEEAGANVVEIGLDRAVVEVDEEAELQKLVEAVEGAGA 51

```

Figure 6. Alignments of WT and mutant CopZ. Figure a. shows the alignment of the section of CopZ cDNA that was mutated. Figure b. shows the alignment of the section of CopZ protein that result from the DNA mutations. Mutations in the sequences are shown in red.

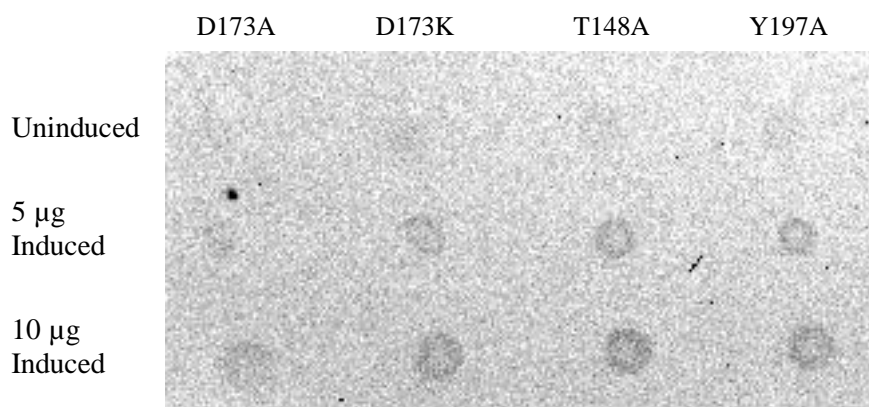


Figure 7. Expression of CopZ mutants. Dot immunoblots of 5 and 10 µg of each protein are shown.

CopZ Expression: Aliquots of induced and uninduced mutant CopZ cultures were blotted onto a nitrocellulose membrane. The His tag on CopZ allowed the membrane to be developed using anti-His antibodies with horseradish peroxidase. The dot blot (Fig. 7) showed that all of the mutants were expressing CopZ.

Purified CopZ: Wild type and mutant CopZ proteins were purified using a Ni-NTA column.

Only D173A and D173K mutants were able to be purified in a large quantity. The S154A and T148A mutants were lost after passing through

the Ni-NTA column. The purified protein was run on a polyacrylamide gel to check for purity (Fig. 8). The approximate yield of each protein was 7 mg/l for WT CopZ, 4.3 mg/l for D173A CopZ, and 3.6 mg/l for D173K CopZ.

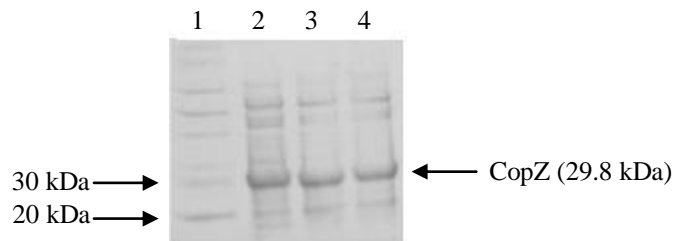


Figure 8. Purified CopZ. Lane 1 is the protein ladder. Lane 2 is WT CopZ. Lane 3 is D173A CopZ. Lane 4 is D173K CopZ.

Loading CopZ with Copper: The copper loading experiment was performed to show that WT CopZ bound 2 Cu^+ ions when loaded stoichiometrically. This experiment confirmed previous results (7). Once CopZ was shown to bind stoichiometrically, the protein did not need to be passed through a sephadex column to remove the excess copper (Data not shown).

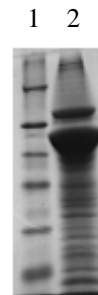


Figure 9. Purified $\Delta\text{N,C-CopA}$. Lane 1 is a protein ladder and Lane 2 is purified $\Delta\text{N,C-CopA}$.

Purification of $\Delta\text{N,C-CopA}$: $\Delta\text{N,C-CopA}$ was purified using a Ni-NTA column. The purified protein (15 μg) was run on a

polyacrylamide gel to check for purity (Fig. 9). The protein was overloaded, which gives the appearance of impurities in the sample. The approximate yield for $\Delta\text{N,C CopA}$ was 1 mg/l.

ΔN,C-CopA ATPase Activity: The ATPase assays were performed to determine if there were any significant changes in CopZ delivery to ΔN,C-CopA when the potential docking residues were altered. First, an experiment to check for differences between the WT and mutant CopZ with respect to the V_{\max} were performed. When compared to WT CopZ protein, the D173A and D173K mutants did not make any noticeable differences to the ΔN,C-CopA V_{\max} (Table 3).

Table 3. Activities of ATPase assays with various forms of 5 μM copper.¹ n = 3, ² n = 2

	Activity (nmol * μg ⁻¹ * h ⁻¹)
CopA + Free Cu ¹	2.08 ± 0.33
WT CopZ ¹	4.79 ± 0.49
D173A CopZ ¹	4.79 ± 0.79
D173K CopZ ²	4.49 ± 0.43

An alteration in the interaction of CopA with CopZ could also result in reduced affinity, which would be indicated by a reduction in the $K_{1/2}$ of the overall catalytic process. In order to address this possibility, additional experiments were done to determine if there was any difference in the $K_{1/2}$ of ΔN,C-CopA when compared to WT CopZ. Initially, the concentrations of CopZ added were 0.5, 1.25, 2.5 and 5 μM. At this concentration, the resolution of the $K_{1/2}$ was low, so additional points with lower concentrations of CopZ were added. These were 0.05, 0.125, and 0.25 μM. The V_{\max} of either mutant of CopZ was slightly lower than that of WT CopZ (Fig. 10). The $K_{1/2}$ of the D173K mutant was slightly lower than the wild type.

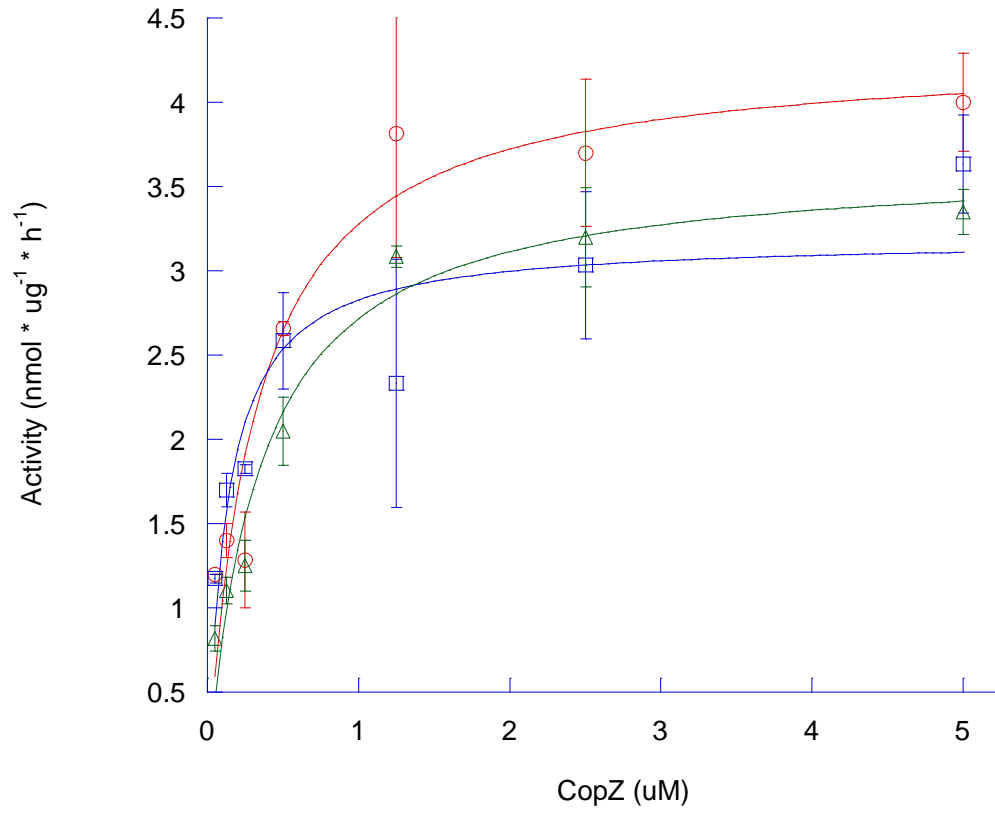


Figure 10. ATPase activity vs. CopZ concentration. The red circles are WT CopZ, the blue squares are D173A CopZ, and the green triangles are D173K CopZ.

Discussion

The homology modeling between the HMA domain of *H. sapiens* superoxide dismutase and *A. fulgidus* CopZ showed that their structures are very similar. The positions of *A. fulgidus* residues can be trusted because key features of the two proteins such as the coordinating cysteines and the alpha helices and beta sheets have the similar locations. This allowed the identification of potential polar residues that dock to CopA during copper delivery.

Not being able to obtain the S154A CopZ mutant was one setback of this project. They multiple PCRs were performed with different procedures to try to get a viable mutation. One problem that this specific mutation may have had was that the primer was not well designed. If this were the case, the primer would not bind to the template with high affinity, leading to no amplification.

Purifying the CopZ mutants was another hinderance. Although the dot blot showed that all the mutants were expressing properly, after passing T148A and Y197A through the Ni-NTA resin, the protein was lost. This may be because somewhere in the purification the His tag or another part of the protein gets cleaved.

There was no change in the V_{\max} of $\Delta N,C$ -CopA ATPase activity when potential docking sites of CopZ are changed. This suggests that the mutation did not affect the rate limiting step of the enzyme. Another alternative explanation is that having only one site mutated is not enough to alter the delivery affinity. Since there are potentially 4 sites, maybe more than one site have to be mutated to see a significant change. That is, each site docks strong enough to overcome the loss of one single site. Another hypothesis is that there are other sites that help CopZ dock to CopA either near the copper coordinating cysteines or on the sides which would allow interactions with

the other regions. These would make any single mutation less severe because there are redundant docking sites.

The ATPase concentration curves had a good fit. There was one point in WT and D173A CopZ that did not fit the curve. This may be due to pipetting error. The $K_{1/2}$ of D173K CopZ was about half that of WT and D173K. The V_{max} of the mutants were slightly lower in the plots than when doing the V_{max} alone. When considering the error in the plot, neither of these differences may be significant.

References

1. Linder, M. C., and Hazegh-Azam, M. (1996) *American Journal of Clinical Nutrition* **63**(5), 797S-811
2. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C., and V. O'Halloran, T. (1999) *Science* **284**(5415), 805-808
3. Argüello, J. M., Eren, E., and González-Guerrero, M. (2007) *Biometals* **20**(3-4), 233-248
4. Argüello, J. M. (2003) *The Journal of Membrane Biology* **195**(2), 93-108
5. Mandal, A. K., Yang, Y., Kertesz, T. M., and Argüello, J. M. (2004) *The Journal of Biological Chemistry* **279**(52), 54802-54807
6. Gonzalez-Guerrero, M., Eren, E., Rawat, S., Stemmler, T. L., and Arguello, J. M. (2008) *The Journal of Biological Chemistry* **283**(44), 29753-29759
7. González-Guerrero, M., and Argüello, J. M. (2008) *Proceedings of the National Academy of Science of the United States of America* **105**, 5992-5997
- 8.
9. Lutsenko, S., Tsivkovskii, R., and Walker, J. M. (2003) *Annals of the New York Academy of Sciences* **986**, 204-211
10. Efremov, R. G., Kosinsky, Y. A., Nolde, D. E., Tsivkovskii, R., Arseniev, A. S., and Lutsenko, S. (2004) *The Biochemical Journal* **382**(Pt 1), 293-305
11. Mandal, A. K., Cheung, W. D., and Argüello, J. M. (2002) *The Journal of Biological Chemistry* **277**(9), 7201-7208
12. Sazinsky, M. H., LeMoine, B., Orofino, M., Davydov, R., Bencze, K. Z., Stemmler, T. L., Hoffman, B. M., Argüello, J. M., and Rosenzweig, A. C. (2007) *The Journal of Biological Chemistry* **282**, 25950-25959
13. Arnesano, F., Banci, L., Bertini, I., Ciofi-Baffoni, S., Molteni, E., Huffman, D. L., and O'Halloran, T. V. (2002) *Genome Research* **12**(2), 255-271
14. Pearson, W. R. (1990) *Methods in Enzymology* **183**, 63-98
15. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18**(15), 2714-2723
16. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) *Nucleic Acids Research* **31**(13), 3381-3385
17. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) *Proceedings of the National Academy of Science of the United States of America* **98**(18), 10037-10041
18. Sambrook, J. and Russel, D. (2001) Molecular Cloning third edition, Cold Spring Harbor Laboratory Press
19. Bradford, M. M. (1976) *Analytical Biochemistry* **72**, 248-254
20. Brenner, A. J., and Harris, E. D. (1995) *Analytical Biochemistry* **226**, 80-84
21. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Analytical Biochemistry* **100**, 95-97