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Interactions of Copper Chaperones and Transporters in Sinorhizobium Meliloti

Joshua Daniel Hall
Worcester Polytechnic Institute

JMA MQP

Interactions of Copper Chaperones and Transporters in *Sinorhizobium Meliloti*

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By

Joshua Hall

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Approved:

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Professor José M. Argüello
Biochemistry
WPI Project Advisor

Abstract:

Cu^+-ATPases are a family of enzyme responsible for the transport of Cu^+ across membranes. These ATPases receive Cu^+ from a specific metallochaperone. Five unique CopA Cu^+-ATPases (CopA1a, CopA1b, CopA2a, CopA2b, and CopA3) and two CopZ chaperones ($CopZ_1$ and $CopZ_2$) were previously discovered in *Sinorhizobium Meliloti*. This study sought to determine the specificity of each chaperone through the use of ClusPro software, which predicts protein-protein interaction through electrostatic calculations. The stoichiometric ratio of metals (Cu^+ and Zn^+) was also determined through atomic absorption spectroscopy. $CopZ_1$ was predicted to be specific to CopA2a and CopA2b while $CopZ_2$ is specific to CopA1a and CopA1b. $CopZ_1$ and $CopZ_2$ were each found to bind a single Cu^+ ion and no Zn^+ ions.

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Introduction:

Heavy metals play an essential role in numerous biological processes of all organisms. Common uses include structural stability, respiration, collagen maturation, redox reactions, electron transfer, and enzyme catalyzed reactions (1). Roughly one third of all known enzymes are classified as metalloenzymes. These contain tightly bound metal cofactors, which are typically transition metals. These function as electrophiles and serve three major functions: binding to substrates to and orienting substrates, mediating oxidation-reduction reactions through reversible changes in the metal ion's oxidation state, and electrostatically stabilizing or shielding negative charges (1).

However, an excess of free metals is toxic to the cell (2). Heavy metals can catalyze Fenton reactions to convert hydrogen peroxide into dangerous hydroxyl free radicals that can bind to and damage DNA. Although the Fenton reaction is the most well understood mechanism for heavy metal toxicity, it is not the only mechanism that copper causes damage through. Copper has a high affinity for thiolates and can degrade iron-sulfur clusters in vital enzymes (3). The loss of function from these various vital enzymes will impede the biological functions of these organisms. The resulting free iron can then form free radicals that will also catalyze Fenton reactions, causing additional damage to DNA. This has made it evolutionarily imperative for organisms to develop mechanisms to maintain heavy metal homeostasis (2). Mechanisms for maintaining heavy metal homeostasis includes controlled transcription regulation, influx, chelation of metal ions, and efflux of metal ions across the membrane.

Chelating agents are molecules that contain two separate coordinate bonds in order to bind divalent metal ions (1). Organisms regulate the expression of metallothioneins, a class of chelating

protein, to chelate metal ions. In copper homeostasis, transcription is regulated by a sensor complex that is highly selective for Cu^{+} ions (4).

Influx is the mechanism of translocating ions from media to the periplasm and periplasm into the cytoplasm via transport proteins. This is typically achieved through ion channels and P-type ATPases. The exact mechanism of copper influx remains poorly understood (4).

Efflux is a mechanism used to move toxic substances such as heavy metals out of the cell using transport proteins (4). Efflux pumps are located on the cytoplasmic membrane. The efflux system responsible for maintaining copper homeostasis is known as the Cue system. Cue system is a homeostasis system that regulates excess copper. There are four proteins in the Cue system: CopA, CueR, CueO, and Cue P. CueR is a metalloregulator that responds to an elevated cytoplasmic copper concentration. Activation of CueR causes increased expression of CopA, an efflux pump (4). CopA is a Cu^{+}-translocating ATPase that is required for copper resistance. It is an inner membrane protein that translocates cytoplasmic Cu^{+} to the periplasm. CueO is a multi-copper oxidase that exits the cytoplasm through the Tat pathway (4). It oxidizes Cu^{+} to Cu^{2+}, which is less toxic. CueP is a periplasmic copper-binding protein proposed to be important for copper resistance, however its exact function is not fully understood (4).

P-type ATPases are a family of membrane proteins involved in efflux. They accomplish this by hydrolyzing ATP into ADP to energize the reaction (5). All P-type ATPases contain the similar structural features (6). Each contains the membrane (M), phosphorylation (P), nucleotide binding (N), and the actuator (A) domains. The M domain is composed of between 6-10 TM helices. The M domain holds a low degree of homology, however the structure remains similar between ATPases. The largest domain is the P domain that always contains the sequence DKTGT. This sequence is responsible for reversibly phosphorylating during enzyme activity. P-

type ATPases all share the same mechanism for metal transport. This process is known as the E1/E2 cycle.

Initially, the ion (or ions) in the cytoplasm binds to the binding site in the E1 form of the enzyme. ATP also binds and phosphorylation of the P domain occurs resulting in a conformational change that alters the enzyme to the E2P form. In this form, the metal is inaccessible to the cytoplasmic side, causing it to leave from the outside. The enzyme is then dephosphorylated and reverts to its E1 starting position.

Cu^+ ATPases are P-type ATPases that undergo this process. In the E1 conformation, the cytoplasmic facing TM-MBS have high affinity for Cu^+ (6). Copper chaperones such as CopZ deliver Cu^+ to the metal binding cites of CopA (4). After binding two Cu^+, the ATPase is phosphorylated and changes to the E1•P conformation. The enzyme then changes to the E2 and the outward facing TM-MBS has a lower affinity for Cu^+, causing it to be released. It has been hypothesized that apo-CusF selectively interacts with E1•Cu^+ to trap the released metals (6). The proposed catalytic cycle of Cu^+ ATPases can be seen in Figure 1 (6).

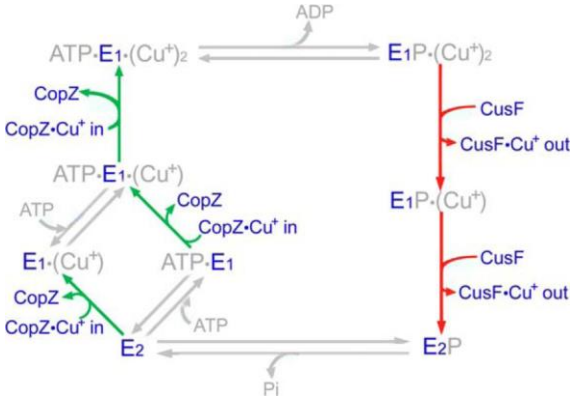


Figure 1: The proposed Cu^+ ATPase catalytic cycle. E1, E1P, E2, and E2P are the major conformations. In the E1 state, the ATP is hydrolyzed and the protein changes to the E1P state. It undergoes a conformational change and is inaccessible to the cytoplasmic side. Cu^+ then removed by CusF, changing the enzyme to the E2P state. The P_i is removed, converting it into the E2 state. Cu^+ is delivered by CopZ to the enzyme in the E2 state, converting it into E1. (6)

P_{1B}-type ATPase, sub-family transport heavy metal ions such as Cu^{+}, Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} via ATP hydrolysis (7). P_{1B-1} ATPases specifically transport Cu^{2+} ions. The structure of P_{1B-1} ATPases contain 6 or 8 transmembrane fragments. It contains two Cu^{+} binding sites. These sites are both used to transport metal across the membrane. Site I consists of two Cys in TM6 and a Tyr in TM7. Site II consists of an Asn in TM7 and a Met and Ser in TM8 (7). The platform is where the chaperone containing Cu^{2+} interact with the ATPase. The Cu^{2+} is then delivered to the ATPase via ligand exchange. The Cu^{2+} interacts with the residues on the TM-MBS until ATP hydrolysis drives the reaction.

P_{1B-1} ATPases specifically transport Cu^{+}, however different ATPases play distinct functional roles. Most confer copper tolerance by driving cytoplasmic metal efflux (8). CopA1 serves this function and is expressed in response to high cytoplasmic Cu^{+} concentrations. Certain P_{1B-1} ATPases such as CopA2 are expressed in association with cytochrome c subunits (8). CopA2 plays no role in Cu^{+} tolerance.

Five homologous Cu^{+}-ATPaese present in *Sinorhizobium meliloti* were previously categorized (9). Different levels of gene expression were detected by exposing the *S. meliloti* mutant strains under different levels of Cu^{+}, oxygen, or nitrosative stress. An increase in CopA1a was observed with increased Cu^{+} concentrations suggesting that it is confers copper tolerance by driving metal efflux (9). CopA1b levels also increased with Cu^{+} concentrations, meaning that it also is expressed to manage copper toxicity. CopA2a and CopA2b were found to be necessary for the assembly of two cytochrome c oxidases. CopA3 is involved in Cu^{+} tolerance. It is regulated by redox stress and required for symbiosis.

However, it is not known how cytosolic copper is targeted to specific ATPases (10). Because copper is toxic inside of the cell, it is bound to a metallochaperone or small peptide. CopZ,

a Cu^+ chaperone found in *A. fulgidus*, delivers copper to Cu^+-ATPase (7). CopZ has been found to bind one Cu^{2+} ion. Two homologous CopZ proteins, $CopZ_1$ and $CopZ_2$ have been identified in *S. meliloti*. It is possible that each CopZ targets specific ATPases. The specificity of each chaperone to each specific ATPase is currently unknown. Chaperones also have the potential to bind to other metal cations with the same charge, although much less strongly.

CopZ contains a CXXC binding site that allows it to take in Cu^+ (7). CopZ also contains three negatively charged patches. One of these patches was found to be necessary for Cu^+ transfer and the subsequent ATPase activation. Bioinformatic analysis shows that the electrostatic docking of CopZ to CopA relies on specific interaction between the E190/193/E205/D145 of CopZ and electropositive CopA (11). CopA has an electropositive platform formed by a kink in the ATPase (7). CopZ has an electronegative surface that allows it to interact with the ATPase. This allows CopZ to participate in ligand exchange with three invariant ATPase residues: Met, Glu, and Asp. These mobilize the Cu^+ from the chaperone to the TM-MBS (7). CopZ binding to the docking site in CopA can be seen in Figure 2 (4).

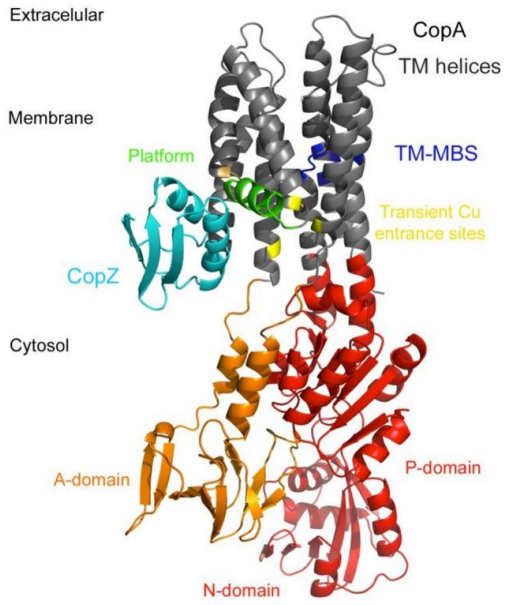


Figure 2: C-terminal binding Cu^+ domain of CopZ modeled using Enterococcus hirae CopZ (Protein Data Bank code 1CPZ). A. fulgidus CopA was used as a template (Protein Data Bank code 3RFU). The transmembrane segments (TM) are in gray, the Cu^+ entrance site aminoacids are in yellow, the transmembrane metal binding sites (TM-MBS) are in dark blue, the actuator in orange, and the nucleotide (N) binding and (P) phosphorylation domain in red.

The objectives of this project were to test which metal ions $CopZ_1$ and $CopZ_2$ bind to and determine their metal binding affinity stoichiometry of each metal. Also, to perform silico analysis for protein-protein electrostatic interactions to determine the specificity of CopZ and CopA for copper exchange.

Materials and Methods:

Cloning and Transformation: $CopZ_1$ and $CopZ_2$ cDNA were cloned and amplified from *S. Meliloti* DNA using the primers listed in Table 1. The cDNA was ligated using the ligation enzyme Bal1 into the pPRIBA1 vector, which confers a C terminal Strep-tag and ampicillin resistance. The plasmids were transformed into *Escherichia coli* BL_{21} (DE_3) cells.

Table 1. Primers used to create amplify CopZ plasmids

Primer	Sequence (5'-3')
$CopZ_1$ (Sma 1009) Forward	AACAGAATTCCACGAGGAGACCATGTATCAC
$CopZ_1$ (Sma 1009) Reverse	TTGTCCATGGGCTGCCGCTGAAATGGCGAC
$CopZ_2$ (Sma 20560) Forward	AACAGAATTCCAAAGGAGACTTTCGATGTAT
$CopZ_2$ (Sma 20560) Reverse	TTGTCCATGGTACCCGGGATAATCGACGTG

Cell Culture and Induction: The transformed *Escherichia coli* cells were grown aerobically at 37°C at 200 rpm in LB media (10 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl) with 100 µg/ml ampicillin. Cells were induced at OD 0.6, with 0.5mM IPTG for 4 hours.

Preparation of Strep-Tactin Resin for Purification of Chaperones: Strep-Tactin Sepharose resin was loaded in an appropriate size column and washed with 5 volumes of deionized H_2O. The resin was equilibrated by passing 5 volumes of Buffer W (100 mM Tris-HCl, pH 8.0, and 150 mM NaCl). The resin was regenerated by washing three times with 5 volumes of Regeneration Buffer and extensively washing the resin with Buffer W (15-20 volumes) (12).

Protein Purification: The cells were harvested by centrifugation at 3200xg for 10 min (12). The cells were washed by resuspending the pellet in 5 volumes of Buffer W (100 mM Tris-HCl, pH 8.0, and 150 mM NaCl) and centrifugation at 3200xg for 10 min. The pellet was resuspended in 5 volumes of Buffer W. The cells were disrupted by sonication 6 times for 30 s, placing the samples on ice for 30 s between repeats. The cell lysate was removed by centrifugation at 8000xg for 30 min. The supernatant was transferred to a fresh tube and the pellet was discarded. The supernatant was loaded into a pre-equilibrated Strep-Tactin column (1 mL per 50-100 nmol of tagged protein). The column was washed with 6 volumes of Buffer W. The protein was eluted with 3-4 column volumes of Buffer E (100 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and 2.5 mM desthiobiotin). 6-8 elution fractions were collected of 1 mL each. The fractions containing the chaperone were identified by SDS-PAGE and combined. The buffer was exchanged by diluting the sample one in five with Buffer C (25mM Tris-HCl, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 0.01% asolectin, and 1 mM phenylmethylsulfonyl fluoride) and concentrated in a 3kDa molecular weight cut-off Centricon. This step was repeated 3-5 times. The protein was concentrated to 1 mg/mL or higher, measured, aliquoted, and stored in 20% glycerol in Buffer C at -20% °C. The fractions containing protein were identified via SDS-PAGE and then combined. The sample was diluted one in five with Buffer C (25mM Tris-HCl, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 0.01% asolectin, and 1 mM phenylmethylsulfonyl fluoride) and concentrated in a 3 kDa molecular weight cut-off centricon. The protein was concentrated to 1 mg/ml determined by Bradford protein assay, and then stored in 20% glycerol at -20°C (12).

Protein Quantification: Protein concentrations were determined by Bradford Assay using Coomassie Brilliant Blue G-250 to stain proteins. 10 µl protein samples and bovine serum albumin (BSA) standards (0, 5, and 10 µg) were mixed and incubated with 1000 µl Coomassie Reagent for 10 min at RT. Thereafter, absorbance was measured at 595 nm (DU 640 Spectrophotometer, Beckman). Protein standards were obtained by diluting a 1 mg/ml stock solution of BSA. Linear regression was used to determine the actual protein concentration of each sample.

SDS PAGE Electrophoresis: One-dimensional SDS polyacrylamide electrophoresis (SDS-PAGE) was carried on 10% SDS polyacrylamide gels. Protein samples were mixed with 6X Laemmli sample buffer (375 mM Tris-HCl pH 6.8, 50% Glycerol, 9% SDS, 0.03% Bromophenol Blue, β-Mercaptoethanol 5%) and were finally heated at 95°C for 5 min. Samples were separated using the Mini-PROTEAN 3 electrophoresis module (Bio-Rad). Electrophoresis was run at 20 mA for 60 min.

Protein Immunodetection: Following electrophoresis, proteins were transferred to nitrocellulose membrane in 1X transfer buffer (25 mM Tris, 192 mM glycine). Each blotting sandwich was prepared in a box filled with the same buffer as follows: cathode - pad - blotting paper - separating gel (from SDS-PAGE) - membrane - blotting paper - pad - anode. The membrane and protein gel were equilibrated with transfer buffer 5 min before starting. Sandwiches were mounted on the Mini Trans-Blot® system (Bio-Rad,) and the chamber was filled with ice-cold transfer buffer. Transfers were performed at 4°C at 370 mV for 70 min. After the transfer, the membrane was equilibrated with PBS containing 5% BSA for 45 min. The membrane was washed 3 times with PBS. Afterwards, the membrane was incubated with the Strep-Tactin antibody at RT for 30 min. The

membrane was washed 3 times with PBS. The membrane was exposed to ECL solution (Thermo Scientific) and visualized using Bio-Rad Gel/Chemi Doc.

Metal Removal from Protein: To convert CopZ to apo form, purified samples were brought to a final volume of 10 mL buffer W. 100mM EDTA and DTT was added. Solution was incubated for 10 min with gentle agitation. The volume was raised to 15 mL and the EDTA was removed by centrifugation in a 3 kDa cut-off centricon at 5000xg. The volume was reduced to 1 mL. The volume was then raised to 15 mL and repeated twice.

Metal Loading to Protein: Metal (Cu^+ or Zn^+) was loaded to apo CopZ by combining CopZ and metal in stoichiometric amounts in a buffer containing 25 mM Hepes (pH 8.0), 150 mM NaCl, and 10 mM ascorbate for 10 min at room temperature with gentle agitation. The unbound metal was removed by centrifugation in a 3-kDa cut off Centricon and the sample was washed with 5 volumes of deionized water. Metal content was measured by furnace atomic absorption spectroscopy (PerkinElmer). To this end, the samples were mineralized with 100 mL of HNO_3 (trace metal grade) for 1 hour at 80°C and then overnight at 20°C. Digestions were concluded after the addition of 25 µL of 30% H_2O_2 and diluted with deionized water.

Homology Docking Modeling: CopA1a, CopA1b, CopA2a, CopA2b, and CopA3 were modeled using LpCopA (Protein Data Bank entry 3RFU) as a template using Swiss-Model (13). $CopZ_1$ and $CopZ_2$ were modeled using EhCopZ (Protein Data Bank entry 1CPZ) as a template. ClusPro modeled the docking interaction of CopZ to CopA using electrostatic predictions. These were then visualized with PyMol (Delano Scientific).

Results:

CopZ Expression: Aliquots of induced $CopZ_1$ and $CopZ_2$ cultures were blotted on a nitrocellulose membrane. The Strep tag on $CopZ_1$ and $CopZ_2$ allowed the membrane to be developed using Strep-Tactin antibodies. The dot blot in Figure 3 showed that both CopZ samples were expressing.

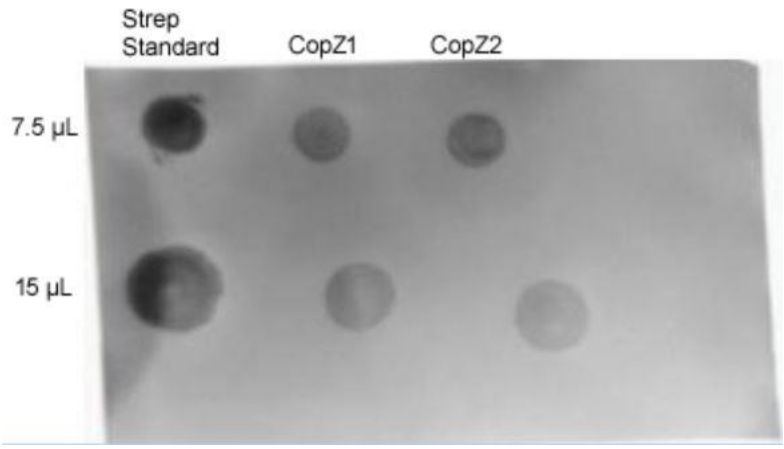


Figure 3: Expression of $CopZ_1$ and $CopZ_2$ samples. Dot immunoblots of 7.5 and 15 µL of media are shown. Previously purified $CopZ_1$ was used as positive control.

Purified CopZ: $CopZ_1$ and $CopZ_2$ proteins were purified using a Strep-Tactin sepharose column. 10 µg of $CopZ_1$, $CopZ_2$, and previously purified $CopZ_1$ (used as a positive control), were run on a polyacrylamide gel to check for purity. Each sample was duplicated on the same gel to perform both a western blot and Coomassie brilliant blue staining. The gel shows that there is a large protein concentration that is 7kD in size, and the western blot clearly shows the Strep-Tag. The approximate yield was 555 µg $CopZ_1$ and 2085 µg $CopZ_2$. The gel and western blot are compared in Figure 4.

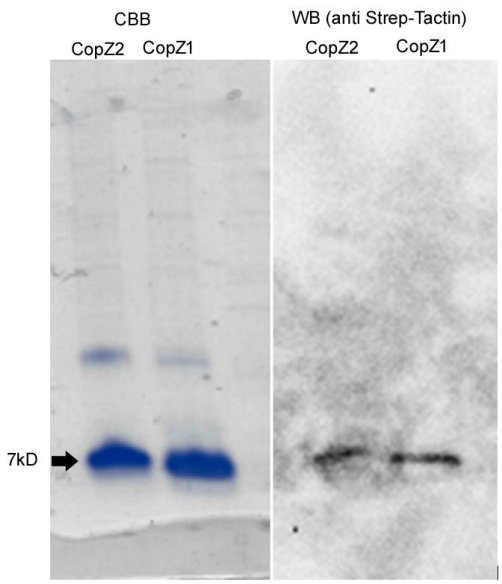


Figure 4: Purified $CopZ_1$ and $CopZ_2$ analyzed by Coomassie brilliant blue (CBB) and Western blot using Strep-Tactin antibody.

Metal Determination by AAS: The metal loaded CopZ samples were analyzed by Atomic Absorbance Spectroscopy to determine their stoichiometric ratio with each metal. The Cu^+ standards used were 10, 25, 50, and 100 ppb. Each Cu^+ bound sample was initially diluted by 10 fold prior to loading to the AAS. The concentration was further diluted 20 fold due to a high concentration. Each sample was analyzed twice for accuracy, and the experiment was repeated an additional time for statistical significance. The Zn^+ bound samples were diluted 20 fold and run on the AAS in the same way. The Zn^+ standards used were 5, 10, 20, and 40 ppb. It was hypothesized that $CopZ_1$ and $CopZ_2$ bind one Cu^+ ion and would not bind any Zn^+ ions. The data (Fig 5) supports this. $CopZ_2$ was found to have a larger Zn^+ value than expected, which indicates that either $CopZ_2$ has a small binding affinity to Zn^+ or the protein concentration of the $CopZ_2$ (Zn^+) sample was higher than measured.

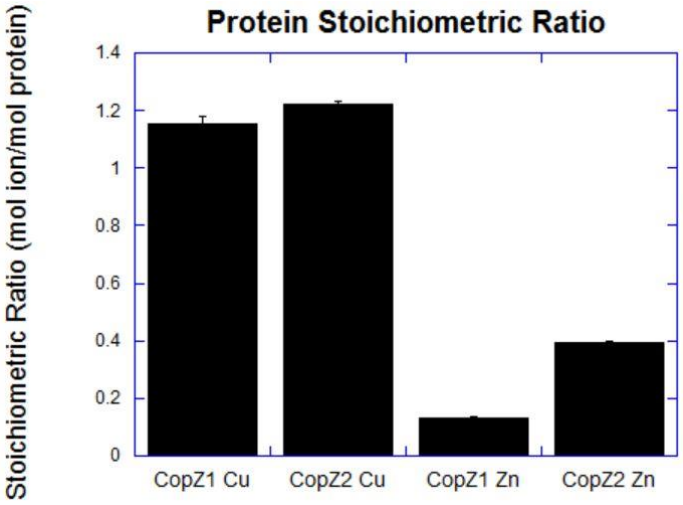


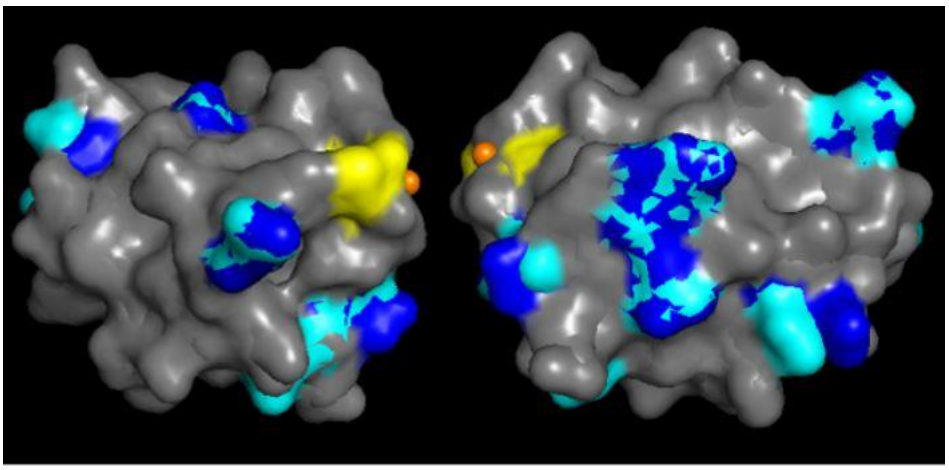
Figure 5: Stoichiometric ratio of each metal binding to each protein. $CopZ_1$ and $CopZ_2$ were measured with Cu^+ and Zn^+

Homology Docking Modeling: The homologous ATPases CopA1a, CopA1b, and CopA3 were previously aligned using ClustalW2 online server at the Conway Institute UCD Dublin (14). The differences in electro-positive residues could correspond to electro-negative residues on the surface of different cytosolic chaperones. The platforms of the ATPases are described in Table 3.

Table 3: Comparison of N-MBD and Platform orientation between CopA1a, CopA1b, and CopA3. Bold and underlined letters are positively charged amino acids present on the platform.

ATPase	N-MBD	Kinck	Platform
CopA1a/CopA1b	CxxC---CxxC	PG	L**R**FF**RK**GVPNLL**R**WTPDM
CopA2a	CxxC---CxxC	PG	**R**FFY**K**SAWNAI**R**HG**R**TNM
CopA2b	CxxC---CxxC	SG	**R**FFY**R**SAWAAL**R**HG**R**TNM
CopA3	CxxC	AG	APFFE**R**AW**R**SLVT**RR**LNM

Two CopZ like proteins were previously identified via BLAST search (15). $CopZ_1$ and $CopZ_2$ were modeled using PyMOL. The electro-negative residues and Cu^+ binding motif can be seen in Figure 6.



.Figure 6: CopZ1 and CopZ2 superimposed. Yellow is the Cu^{+} binding motif. Blue is negatively charged amino acid residues on $CopZ_1$. Cyan is negatively charged amino acid residues on $CopZ_2$. Orange is a copper ion.

The most probable docking position of $CopZ_1$ and $CopZ_2$ was determined using ClusPro protein-protein docking software (16). ClusPro analyzes the positive and negative regions of the protein to estimate the most electrostatically-favored docking position. $CopZ_1$ interacted with CopA2a with a free energy difference of -192.6. CopA2a is homologous to CopA2b, so $CopZ_1$ is likely specific to both. $CopZ_2$ interacted with CopA1a with a free energy difference of -202.5. $CopZ_2$ likely interacts with CopA1b also because they are homologous. No viable interaction prediction was found with CopA3. The predicted interactions can be seen in Figure 7.

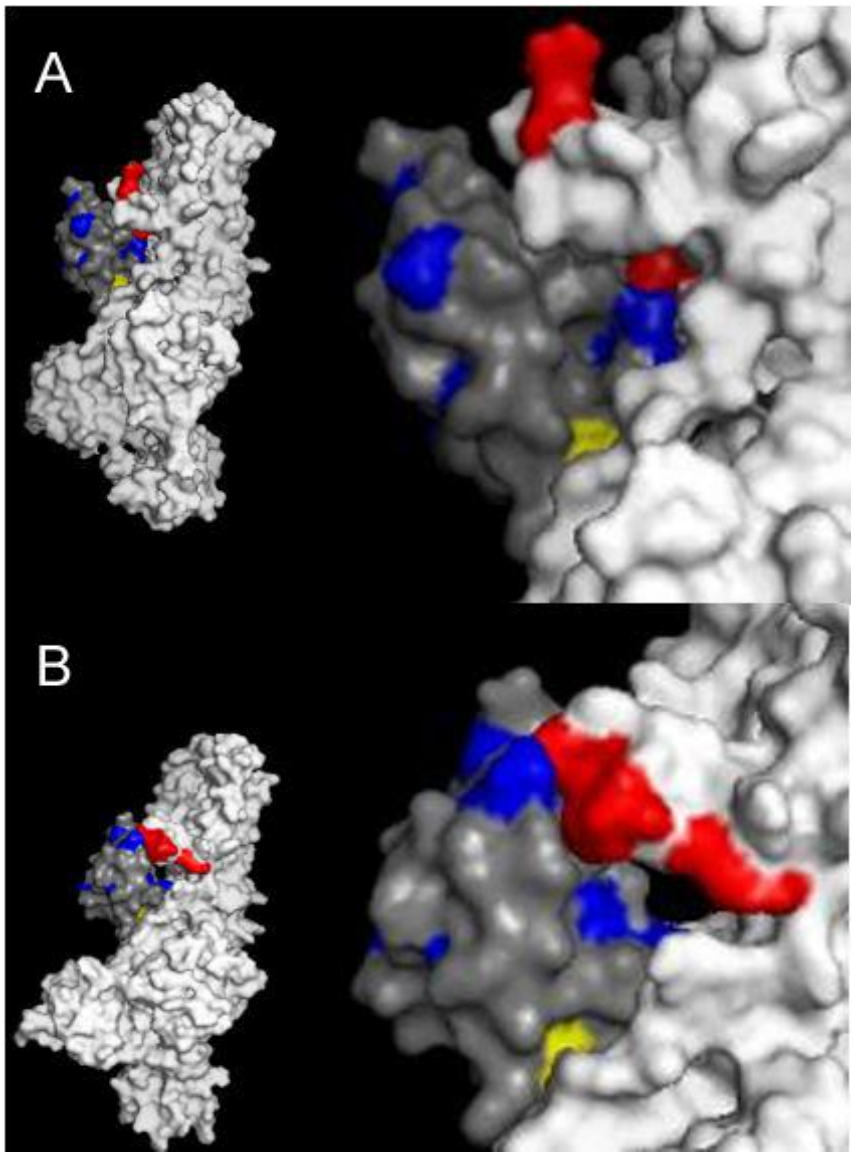


Figure 7: Model of (A) CopA1a and $CopZ_2$ and (B) CopA2a and $CopZ_1$ interaction generated by ClusPro. Positively charged residues on the docking platform (red), negatively charged residues on the chaperone (blue), and copper binding motif (yellow) are highlighted. The chaperones are gray and the ATPases are white.

Discussion:

Atomic Absorption Spectroscopy showed that $CopZ_1$ and $CopZ_2$ each stoichiometrically bind a single Cu^+ ion and no Zn^+ ions. The value of Zn^+ measured in $CopZ_2$ was larger than expected. $CopZ_1$ is homologous to $CopZ_2$ and retained negligible Zn^+, so $CopZ_2$ should have the same value. This indicates the concentration of protein measured was lower than the true value.

$CopZ_1$ is predicted to be specific to CopA2a and CopA2b. $CopZ_2$ was predicted to be specific to CopA1a and CopA1b. This is reasonable as the homologous ATPase pairs have the same docking site. No viable interaction was found between CopA3 and either chaperone. This is likely due a limitation with ClusPro software.

Dimerization of chaperones was a hindrance. This was largely reversed through addition of DTT, however a degree of dimerization was observed, which could have prevented chaperones from functioning properly.

Future studies can verify the predictions of ClusPro experimentally. Cu^+ transfer of each chaperone to each ATPase can be performed to measure the ATPase activity. This will determine if a chaperone is specific to an ATPase.

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