April 2009

Purification and Characterization of P-type ATPases

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Purification and Characterization of P-type ATPases

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

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Hyungjoon Kim

Date: 15 April 2009

Approved:

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Professor José M. Argüello
Biochemistry
Major Advisor
Abstract

P-type ATPases are a family of enzymes responsible for the transport of lipids and ions across membranes. Although models exist for these enzymes, knowledge on specific transporters is more often than not incomplete. This study sought to improve the knowledge of certain Mycobacterium tuberculosis ATPases (ctpF, ctpG, ctpJ) through biochemical characterizations using ATPase activity assays and in vivo effects of the proteins on bacterial metal resistance. In addition, protocols for detergent solubilization, micellar reconstitution, and isolation were optimized. Purified CtpG showed evidence of activity in the presence of copper; however, none of the other proteins’ biochemical studies or in vivo explorations of all three proteins in various E. coli backgrounds yielded clear results on the effect of these proteins on the metal resistance of the bacterium.
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**Introduction**

Heavy metals are elements that have specific weights greater than 5 g/cm$^3$ (1). In organic systems, they are involved in various functions such as redox reactions, electron transfer, enzyme-catalyzed reactions, pigmentation, and structural stability, among others (2, 3). This group of about 40 elements poses an interesting dilemma in that, although they are required at trace amounts, in excess quantities prove to be extremely toxic to the cells (1, 2). As such, organisms have developed mechanisms for maintaining the homeostasis of heavy metals.

One such mechanism is the process of active transport through P-type ATPases. P-type ATPases are membrane proteins which transport a variety of ions by hydrolyzing ATP. Their biological importance is underscored by the fact that they are ubiquitous through multiple kingdoms (4). A phylogenetic analysis of multiple P-type ATPases from different species (Figure 1 (4)) shows alignment based on the ion transported. This indicates that sequence homology between enzymes also indicate functional similarities. This in turn indicates the importance of the structure in the activity of the enzyme. As ubiquitous as they are, however, different ATPases also hold important distinctions which allow them to be categorized into different families. P-type ATPases can be divided into five large families which can be further divided into subtypes (4). Each subtype holds a particular ion specificity. P-IA ATPases are bacterial ion pumps which may be ancestral enzymes. P-IB ATPases transport the “soft” transition metal ions. P-II ATPases are the Ca$^{2+}$, Na$^+$/K$^+$, and H$^+$/K$^+$ pumps. P-III ATPases are those members that maintain a membrane potential – H$^+$ in plants and fungi and Na$^+$/K$^+$ in animals. P-IV ATPases are involved in the maintenance of the lipid bilayer asymmetry. P-V ATPases have substrates that are as yet unidentified (4).
Figure 1: Phylogenetic tree of the P-type ATPase families. The subfamilies cluster according to substrate specificity, even across multiple species. Gene products are color coded by producing species: green – *Arabidopsis thaliana*; orange – *Caenorhabditis elegans*; grey – *Escherichia coli*; dark blue – *Homo sapiens*; light blue – *Methanobacterium thermoautotrophicum*; yellow – *Methanococcus jannaschii*; purple – *Synechocystis PCC6803*; and red – *Saccharomyces cerevisiae*

Structurally, all P-type ATPases share certain common points. Four major domains are ubiquitously present in P-type ATPases: the membrane (M), actuator (A), phosphorylation (P), and nucleotide binding (N) domains. The M domain consists of the 6-10 transmembrane helices and the small inter-transmembrane loops that connect them. Although it is the largest domain of the four primary domains, it also holds a very low degree of homology. Their structures, however, seems to have much more homology than the sequences (4). Depending on the subfamily of the ATPase, certain transmembrane helices contain residues capable of coordinating the ion that is to be transported. The P domain is the largest of the cytoplasmic
domains and is also the most highly conserved domain. The signature sequence DKTGT can be found here, where D is the residue reversibly phosphorylated during enzymatic activity. Integrated in the P domain is the N domain. The N domain is linked to the P domain by a conserved hinge that consists of antiparallel peptide strands. Crystallographic data shows that only the adenosine of the ATP is pocketed by the N domain’s conserved Phe. The phosphates seem to lie in the solvent, which allows interaction with the P domain (4). Upstream of the P and N domains, connected to a different transmembrane helix, is the actuator domain. The role of the N domain in the enzymatic activity is not initially apparent. Experiments show that during activity the A domain interacts with the P and N domains, apparently protecting the phosphoryl group from hydrolysis (4).

Variations between different subfamilies of P-type ATPases arise primarily in the number of transmembrane helices, the precise number and type of residues involved in metal coordination, and the presence of additional cytoplasmic domains. In P-IA ATPases, for example, the M domain consists of 6 transmembrane helices while in P-II ATPases, the M domain consists of 10 transmembrane helices (Figure 2 (5)). P-I ATPases also often contain cytoplasmic metal-binding domains with conserved histidine- cysteine-rich sequences (6). The residues involved in metal coordination are relatively unique for each subfamily but are strongly conserved.
Figure 2: Structure models for (A) Bacterial PIB-ATPase and (B) PII-ATPase. A denotes the actuator domain, P denotes the phosphorylation domain, and N denotes the nucleotide binding domain.

Despite their variations, P-type ATPases all have the same mechanism. Binding of the ion or ions along with the nucleotide leads to a phosphorylation of the P domain. This leads to a conformational change resulting in release of ligands. The hydrolysis of phosphate leads to a conformational change back to the initial state. This activity can be characterized by the E1/E2 cycle (Figure 3 (6)). The E1 form has a high affinity for cytoplasmic metals. It binds metal at a certain stoichiometry along with an ATP. Through the hydrolysis of the ATP and the subsequent phosphorylation of the enzyme, the enzyme undergoes a conformational change into the E2P form. This causes the metal to become inaccessible to the cytoplasmic side. The metal then leaves the enzyme from the outside. When the enzyme is dephosphorylated, it reverts to the E1 form to restart the cycle.

Figure 3: The E1/E2 catalytic cycle. E1, E1P, E2, and E2P are the primary conformations of the enzyme in this cycle. M^{n+} is the metal transported of unknown valency n+. n denotes that the stoichiometry of transfer is unknown. The subscripts of M^{n+} denote the location of the metal (cytoplasmic or extracellular) (6).
*Mycobacterium tuberculosis* is an obligate pathogen which could contain as many as 28 putative metal transporters (7). These metal transporters are most likely crucial to the pathogenesis of *Mycobacterium tuberculosis* since it resides the phagosome (8). The phagosome presents a challenge to pathogens in the form of a harsh environment; it presents extremely low pHs, high metal content, and reactive oxygen species. The harsh environment is created by the fusion of the phagosome with a lysosome. *Mycobacterium tuberculosis* survives in this hostile environment by preventing this fusion and therefore preventing the introduction of harsh chemicals and enzymes. This creates a slightly more tolerable environment that the bacterium lives in. As a consequence of the mechanism of the restriction of lysosome fusing, however, the host phagosome also obtains fewer nutrients from nutrient organelles. This leads to depreciated nutritional levels for the parasite as well. There are implications (that arise from the observations on the NRAMP orthologue Mramp) that essential metal homeostasis is an important factor to the survival of the parasite (7). Investigations into the mechanism and the degree to which these metal transporters play a role in the survival and virulence of *Mycobacterium tuberculosis* should provide insights as to novel drug targets and treatment options for tuberculosis.

Certain metal transporters in the *Mycobacterium tuberculosis* genome draw specific interest. CtpG encodes a putative P-IB ATPase that may transport copper (7). This is of interest because there are homologues like the *Archaeoglobus fulgidus* copA gene, which also is a copper-transporting P-IB ATPase, have already been characterized well. It contains a concensus
CCXXE cytoplasmic metal-binding domain along with an APC in the transmembrane metal-binding domain. The key DKTGT is also apparent in the cytoplasmic P domain.

CtpJ encodes another putative P-IB ATPase. It contains an SPC in the transmembrane metal-binding domain. Sequential comparison with functionally categorized enzymes reveals that ctpJ encodes a putative heavy metal transporter. This means it probably plays a role in the survival mechanisms of Mycobacterium tuberculosis after it has infected the host phagosome.

CtpF is another gene of interest as it is a putative P-II ATPase that transports Ca\(^{2+}\) or Mg\(^{2+}\). This is of particular interest since Mg\(^{2+}\) probably plays an important role in Mycobacterium tuberculosis virulence signaling (7). Even if ctpF is not involved with Mg\(^{2+}\) homeostasis, as a putative P-II ATPase it may fill a niche in maintaining a voltage gradient. This could be of interest as well since part of the survival mechanism in phagosomes result in a milder intra-phagosomal pH (8).

In order to study these genes from a biochemical point of view, it is necessary to isolate the corresponding proteins and characterize their enzymatic activities. This can be done through overexpression in E. coli and isolating each protein through purification steps. Since the different ‘Ctp’s are membrane proteins, it is necessary to include a step which will allow for the gentle emulsification of the membrane using detergents. In order to achieve this, existing purification protocol will be adjusted for optimal detergent type and amount. Optimal purification would result in maximal yield of pure, active enzyme while minimizing procedure costs.
Materials and Methods

Strains used: Multiple *E. coli* strains were used in different growth conditions. Top10 cells were grown in medium containing 1.6% Tryptone, 1% Yeast Extract, and 0.5% NaCl (2x YT medium) with 100 µg/ml of ampicillin. Top10cp cells were grown in 2x YT medium containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. LMG194ΔZntA cells, which lack the zinc transporter ZntA, and BL21DE3ΔCopA cells, which lack the copper transporter CopA, were grown in 2x YT medium containing 100 µg/ml of ampicillin and 20 µg/ml of kanamycin. TKW3205 cells, which lack the kdpFABC operon that encodes a potassium transport system, and TRK2000 cells, which lack both the kdpDE operon and kdpFABC operon, were grown in medium containing 0.5% Yeast Extract, 1% Tryptone, and 1% KCl (KML medium) with 100 µg/ml of ampicillin. All *E. coli* strains were transformed with a pBAD/TOPO vector, which contains an arabinose-inducible promoter and adds a poly-His tag to the C-terminus of the inserted gene. Each strain (Top10cp, LMG194ΔZntA, BL21DE3ΔCopA, TKW3205, and TRK2000) transformed with the empty vector and the vector containing ctpG were provided by a graduate student at the lab for shared use. Each strain was also transformed with vector containing ctpF and ctpJ for the experiments.

Protein expression: Dot blot experiments to determine the expression of the genes inserted in the vectors were performed as follows: Transformed *E. coli* were grown in 5 ml overnight cultures. The overnight cultures were then diluted to OD<sub>600</sub> 0.1 in 5 ml of the appropriate medium and incubated further at 37°C with agitation. When the cells had grown to OD<sub>600</sub> 0.6, 1 ml aliquots were saved and pelleted. The rest of the culture was induced with 0.02% final concentration of L-arabinose (Sigma) and incubated. 3 hours after induction, 1 ml of the culture was saved and
The pellets were resuspended in 90 µl of water and sonicated at 40 Watts for 30 to 60 seconds. SDS was added to a final concentration of 0.1% and the samples were boiled for 5 minutes. 10 µg of each sample was blotted on a nitrocellulose membrane along with 5 µg of BSA as negative control and 5 µg of purified His-tag ctpC as a positive control. The membrane was dried thoroughly before being soaked in buffer containing 50 mM Tris pH 7.4, and 0.2 M NaCl (Buffer 229). The membrane was blocked with Buffer 229 + 5% w/v nonfat dry milk (blocking solution) for 20 minutes. The blocked membrane was rinsed in Buffer 229 for 3×5 minutes before being subjected to incubation with primary antibody (rabbit anti-His, 1:5000 dilution in blocking solution) for 2 hours. The membrane was then rinsed with Buffer 229 + 0.05% Tween-20 for 2×5 minutes before another rinse in Buffer 229 for 5 minutes. After the rinse, the membrane was subjected to incubation with secondary antibody (goat anti-rabbit HRP conjugate, 1:5000 dilution in blocking solution) for 1 hour. The membrane was rinsed with Buffer 229 + 0.05% Tween-20 for 2×5 minutes and with Buffer 229 for 5 minutes before exposure to ECL solution (Thermo scientific) and capture by Bio-Rad Gel/Chemi Doc.

Optimization of protein solubilization: LMG194 cells harboring a pCRT7/NT-TOPO plasmid containing the Archaeoglobus fulgidus copA gene were grown. At OD$_{600}$ 0.6 the cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for a further 3 hours with agitation. The cultures were centrifuged at 4000 rpm with the Sorvall GSA rotor for 20 minutes to collect the cells. The cell pellets were resuspended in 10 ml buffer containing 25 mM Tris pH 7.0, 100 mM sucrose, and 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Buffer A). The cell suspension was lysed by passage through a French Pressure Cell at 10,000 psi three times. The lysate was centrifuged at 9000 rpm with the Sorvall GSA rotor for 30 minutes. The
supernatant was collected and centrifuged at 33,000 rpm in the Beckman 50.2 Ti rotor for 1 hour to collect membrane proteins. The pellet was resuspended in buffer containing 25 mM Tris pH 8.0, 100 mM sucrose, 500 mM NaCl, and 1 mM PMSF (Buffer B) and homogenized using a glass hand homogenizer. The homogenized protein was diluted to 3 mg/ml in Buffer B, and aliquots of the same quantified amounts of protein were subjected to solubilization under different detergent conditions: 0.75% n-dodecyl-β-maltoside (DDM) as a control, based on previous lab protocol; 0.25% decaethylene glycol monododecyl ether (C12E10); or 0.5% C12E10; 0.75% C12E10; 1% C12E10. Solubilized samples were centrifuged at 33,000 rpm in the Beckman 50.2 Ti rotor for 1 hour. The protein in both the supernatant and pellet were quantified separately. Based on the results, another experiment was done with the following solubilization conditions: 0.75% DDM as a control; 1% C12E10; or 0.25% DDM, 0.5% C12E10 mix. Again, quantified aliquots of membrane were subjected to each condition, centrifuged at 33,000 rpm for 1 hour, and the protein in the pellet and supernatant were quantified. Protein concentration determination was done by the method described by Bradford (9).

*CtpG expression and purification:* Top10cp cells harboring a pBAD/TOPO plasmid containing the ctpG gene were grown. At OD$_{600}$ 0.6, the cells were induced with 0.02% L-arabinose and incubated for another 3 hours with agitation. Then, the entirety of the cell culture was centrifuged at 4000 rpm with the Sorvall GSA rotor for 20 minutes to collect the cells. The cell pellets were resuspended in 10 ml Buffer A. The cell suspension was lysed by passage through a French Pressure Cell at 10,000 psi three times. The lysate was centrifuged at 9000 rpm in the Sorvall GSA rotor for 30 minutes. The supernatant was collected and centrifuged at 33,000 rpm.
with the Beckman 50.2 Ti rotor for 1 hour to collect membrane proteins. The pellet was resuspended in Buffer B and homogenized using a glass hand homogenizer. The homogenized protein was diluted to 3 mg/ml in Buffer B and solubilized by adding 0.25% DDM and 0.5% C12E10. The solubilization was done with stirring at 4°C for 30 minutes. The solubilized mixture was centrifuged at 33,000 rpm in the Beckman 50.2 Ti rotor for 30 minutes. The supernatant was incubated with Ni²⁺-nitrotriacetic acid (Ni-NTA) slurry equilibrated with Buffer B + 5 mM imidazole for 1 hour with rocking at 4°C. The slurry was packed in a column and washed with 10 volumes of Buffer B + 5 mM imidazole and with 10 volumes of Buffer B + 20 mM imidazole. The protein was eluted with 10 volumes of Buffer B + 150 mM imidazole. The eluate was collected and concentrated using a 30 kDa-cutoff centricon. The imidazole was diluted out by resuspension in buffer containing 25 mM Tris (pH 8.0), 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 1 mM dithiothreitol (DTT) and further concentration. Glycerol was added to a final concentration of 10% and purified protein was aliquoted and stored at -20°C until use. Protein concentration determination was done by the method described by Bradford (9).

Activity assay of copA and ctpG: For A. flugidus CopA, activity assays were performed in buffer containing 50 mM Tris (pH 7.5), 3 mM MgCl₂, 3 mM ATP, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 2.5 mM DTT, 2.5 mM cysteine, 2.5 µg of purified CopA, and excess Cu²⁺. The mix was incubated at 75°C for 10 minutes before the reaction was stopped and free Pi was determined by the method described by Lanzetta et al. (10). The activity assay for CtpG was the same as that of CopA except: the buffer contained only 25 mM NaCl instead of 400 mM NaCl, and the mix was incubated at 37°C rather than 75°C.
**Metal sensitivity test:** Plates containing 2x YT + 2% agar + 0.02% arabinose + 100 µg/ml of ampicillin were prepared. 0.3 ml of overnight cultures of Top10 containing empty pBAD vector, pBAD vector + ctpF, pBAD vector + ctpG, and pBAD vector + ctpJ was vortex mixed into 3 ml of top agar containing 2x YT + 0.7% agar + 0.02% arabinose + 100 µg/ml of ampicillin and spread over the prepared plates. The cells were allowed to express the appropriate proteins for 1 hour. Small filter paper discs (radius ~0.4 cm) were soaked with 10 µl of various metal dilutions and placed on top of the plates. The bacteria were allowed to grow for a further 5 hours before their pictures were taken. The same test was done on LMG194 ΔZntA and BL21DE3 ΔCopA cells, except that the plates and top agar also contained 20 µg/ml of kanamycin.

Plates containing KML + 2% agar + 0.02% arabinose + 100 µg/ml of ampicillin were prepared. Also, plates containing less KCl (0%, 0.25%, 0.5%, 0.75%) were prepared. Overnight cultures of TKW 3205 containing empty pBAD vector, pBAD vector + ctpF, pBAD vector + ctpG, and pBAD vector + ctpJ were grown. The overnight cultures were diluted to OD$_{600}$ 0.5 and serial dilutions were made at $10^{-1}$ to $10^{-9}$. 5 µl of each dilution was placed on the plate, with each plate containing both TKW 3205 + pBAD and one of TKW 3205 + ctpF, ctpG, or ctpJ. The plates were incubated for 24 hours before their pictures were taken.
Results

In order to ascertain the role of each protein, their specific activities can be determined. Since it appears that their role is to aid in the homeostasis of metals and because they are ATPases, the specific metal they transport should be found. Once it can be demonstrated that the proteins are active in the presence of certain metals, they can be analyzed in a living system. If their role is to confer resistance to the metals, the bacteria transformed with the genes coding for these proteins should show different growths in high metal conditions compared with those that were not transformed.

Protein expression: In order to begin the experiments, it had to be confirmed that the various cells expressed the proteins of interest. Since these contained a C-terminus poly-His tag, a Western blot following the proper expression allowed for this confirmation.

First, expression of CtpG was checked for (figure 4):

Figure 4: Results of a dot blot experiment for various cell lines containing the pBAD / ctpG plasmid. The area above label (a) is the negative control. The dot above label (b) is the positive control. Dots labeled 1-8 are, respectively:

(1) ΔCopA, 0 hr expression; (2) ΔZntA, 0 hr expression; (3) TKW3205, 0 hr expression; (4) ΔCopA, 3 hr expression; (5) ΔZntA, 3 hr expression; (6) TKW3205, 3 hr expression; (7) TRK2000, 0 hr expression; (8) TRK2000, 3 hr expression
The darkened circles represent the presence of localized antibodies, which in turn represent the presence of a His-tagged protein. Since there are notable differences between the 0 hour expressions and 3 hour expressions of the same cell culture, it can be said that the protein is induced by the applied inducer.

After CtpG’s presence was confirmed, the same check was performed for CtpF and CtpJ (figure 5):

![Figure 5: Results of a dot blot experiment for various cell lines containing either the pBAD / ctpF plasmid or the pBAD / ctpJ plasmid. The area above label (a) is the negative control. The dot above label (b) is the positive control. Dots labeled 1-16 are, respectively:

(1) ΔCopA ctpF, 0 hr expression; (2) ΔZntA ctpF, 0 hr expression; (3) TKW3205 ctpF, 0 hr expression; (4) TRK2000 ctpF, 0 hr expression; (5) ΔCopA ctpF, 3 hr expression; (6) ΔZntA ctpF, 3 hr expression; (7) TKW3205 ctpF, 3 hr expression; (8) TRK2000 ctpF, 3 hr expression; (9) ΔCopA ctpJ, 0 hr expression; (10) ΔZntA ctpJ, 0 hr expression; (11) TKW3205 ctpJ, 0 hr expression; (12) TRK2000 ctpJ, 0 hr expression; (13) ΔCopA ctpJ, 3 hr expression; (14) ΔZntA ctpJ, 3 hr expression; (15) TKW3205 ctpJ, 3 hr expression; (16) TRK2000 ctpJ, 3 hr expression.

Similar to the CtpG in figure 4, CtpF and CtpJ can be said to be expressed in the cell cultures investigated. Since the appropriate cell backgrounds express the appropriate proteins, it was time to develop a scheme for purifying the desired His-tagged membrane proteins.

*Optimization of protein solubilization:* Previous protocols developed in our laboratory used DDM to solubilize membrane proteins (11). An alternate method using C12E10 was explored.
First, a standardized curve of solubility was constructed using *A. fulgidus* CopA. The solubilization offered by various concentrations of C12E10 was compared against a DDM solubilization control. The solubilized mixture was centrifuged in the Beckman 50.2 Ti rotor at 33,000 RPM to separate solubilized protein from unsolubilized protein. The total amount of protein used per condition was 9 mg. The protein in either fraction was quantified and the results are tabulated in Table 1:

**Table 1: Solubilization of protein by varying concentrations of C12E10**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Solubilized</th>
<th>Unsolubilized</th>
<th>Total</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% C12E10</td>
<td>4.96 mg (55.11%)</td>
<td>3.56 mg (39.56%)</td>
<td>8.52 mg</td>
<td>94.67%</td>
</tr>
<tr>
<td>0.75% C12E10</td>
<td>4.71 mg (52.33%)</td>
<td>3.04 mg (33.78%)</td>
<td>7.75 mg</td>
<td>86.11%</td>
</tr>
<tr>
<td>1.0% C12E10</td>
<td>4.96 mg (55.11%)</td>
<td>3.05 mg (33.89%)</td>
<td>8.01 mg</td>
<td>89.00%</td>
</tr>
<tr>
<td>1.25% C12E10</td>
<td>4.80 mg (53.33%)</td>
<td>3.04 mg (33.78%)</td>
<td>7.84 mg</td>
<td>87.11%</td>
</tr>
<tr>
<td>0.75% DDM</td>
<td>7.21 mg (80.11%)</td>
<td>1.21 mg (13.44%)</td>
<td>8.42 mg</td>
<td>93.56%</td>
</tr>
</tbody>
</table>

As the table shows, the solubilization produced by C12E10 at the concentrations tested was considerably less than that produced by DDM. Since the original goal was to reduce the cost of each experiment while retaining good solubility, a refined procedure using a lower concentration of DDM combined with C12E10 was tested. The total amount of protein used per condition was 1 mg. The solubilized and unsolubilized protein were quantified and tabulated in Table 2:

**Table 2: Solubilization of protein by varying concentrations of DDM**

<table>
<thead>
<tr>
<th>DDM</th>
<th>C12E10</th>
<th>Solubilized</th>
<th>Unsolubilized</th>
<th>Total</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1%</td>
<td>0.5%</td>
<td>414.3 µg (41.43%)</td>
<td>309.9 µg (30.99%)</td>
<td>724.2 µg</td>
<td>72.42%</td>
</tr>
<tr>
<td>0.25%</td>
<td>0.5%</td>
<td>641.9 µg (64.19%)</td>
<td>208.8 µg (20.88%)</td>
<td>850.7 µg</td>
<td>85.07%</td>
</tr>
<tr>
<td>0.5%</td>
<td>0.5%</td>
<td>652.6 µg (65.26%)</td>
<td>192.6 µg (19.26%)</td>
<td>845.2 µg</td>
<td>84.52%</td>
</tr>
</tbody>
</table>
As shown in the data, there is little difference between 0.25% DDM, 0.5% C12E10 and 0.5% DDM, 0.5% C12E10. 0.25% DDM, 0.5% C12E10 was chosen as the new solubilization condition for membrane proteins.

*CtpG expression and purification:* Using the new solubilization protocol, CtpG was purified from membranes of Top10 cells expressing the protein. In order to check which fraction CtpG was eluted, Ni-NTA column fractions were analyzed by SDS-PAGE.

**Figure 6:** The first 12 fractions of elution. First lane is a protein marker. Second lane is the flowthrough after affinity binding. Third through eighth lanes are 5 mM imidazole buffer elutions, in order of elution. Ninth through twelfth lanes are 20 mM imidazole buffer elutions, in order of elution.

**Figure 7:** The last 9 fractions of elution. First lane is a protein marker. Second through seventh lanes are 150 mM imidazole buffer elutions, in order of elution. Eighth through eleventh lanes are 300 mM imidazole buffer elutions, in order of elution.

Of these, the last five 20 mM imidazole buffer fractions, all 150 mM imidazole buffer fractions, and the first three 300 mM imidazole buffer fractions were collected and concentrated. The purity of the combined protein was determined with another SDS-PAGE before moving on to measure the activity of the protein.
Before the activity of CtpG could be analyzed, however, the activity of *Archaeoglobus fulgidus* CopA, which is known, had to be measured to make sure that the new solubilization scheme did not affect the enzymatic activity of the purified protein. In order to test this, CopA was prepared with both the old solubilization protocol using only DDM and the new solubilization protocol using DDM and C12E10, and the resulting protein was purified separately.

Afterwards, both samples were subjected to the copper ATPase activity assay outlined in Materials and Methods. After 3 measurements, the activity was calculated to be $2.4 \pm 0.4 \text{ nmol} \mu\text{g}^{-1} \text{ h}^{-1}$ for the DDM solubilized CopA and $2.6 \pm 0.5 \text{ nmol} \mu\text{g}^{-1} \text{ h}^{-1}$ for the C12E10/DDM solubilized CopA. Since the two numbers are within standard error of each other and are similar...
to published values for CopA velocity (12), it was decided that the C12E10/DDM solubilization had no significant effect on the activity of the protein.

Using this information, it was possible to continue with the investigation of CtpG activity using only the C12E10/DDM solubilized CtpG. When the experiment was done under the same conditions, calculations showed the activity of CtpG to be $1.5 \pm 0.4 \text{ nmol} \mu\text{g}^{-1} \text{h}^{-1}$.

Afterwards, both CtpF and CtpJ were purified to check for their activities in the presence of metals.

![Figure 10: Purified CtpF](image)

![Figure 11: Purified CtpJ](image)

The experiments were done with CtpF and CtpJ using the metals copper, nickel, magnesium, cobalt, iron, cadmium, sodium, and potassium, but no significant activity could be determined. This could be due to many factors, including incorrect pH used during the activity assay, presence of inhibitors in the enzyme mixture, and the enzymes not being specific transporters of the tested ions. Even though the activities of CtpF and CtpJ were not known, metal resistance experiments were done with the two proteins to see if the proteins had an effect on the resistance of the bacterium against certain metals since the conference of metal resistance would help in determining what ion each protein transports.
Metal sensitivity test: After the activity of the protein had been established, its effect on the *in vivo* system was explored with the metal sensitivity tests outlined in materials and methods. When the diameter of the inhibition halos or the number of colonies on the drop tests were compared to get a sense of the cells’ ability to survive a high metal environment, no significant differences were found. Cells transformed with each gene were subjected to cobalt, copper, iron, magnesium, manganese, nickel at the following concentrations: 1 µM, 10 µM, 100 µM, 1 mM, 10 mM, 100 mM, 250 mM, 500 mM, 750 mM, 1 M during the inhibition halo experiment described in the Materials and Methods. The experiment was repeated a total of four times with each strain-protein combination (data not shown). In addition, the effect of each protein on potassium resistance was explored through the potassium drop test experiment described in the Materials and Methods. The experiment was repeated a total of four times with each strain-protein combination (data not shown).
Discussion

Biochemical analysis showed that CtpG is active in the presence of copper. Since CtpG appears to be a P-IB ATPase (7), this suggests that copper is a substrate of CtpG. For confirmation, however, a direct measurement of copper disappearance or appearance on either side of an intact membrane would be beneficial.

In vivo, however, cells with the pBAD/ctpG insert showed no change in resistance to copper. Since this was true even of E. coli lacking CopA (a copper transporter), it may be due to the fact that ctpG is not directly involved in copper transport, ctpG is not localized to the proper locations, or that there exist other mechanisms for the tolerance of copper in the bacteria.

Neither CtpF nor CtpJ showed any activity in the presence of the metals tested. It may be possible that the ATPase assay conditions might not have been ideal for CtpF and CtpJ. Altering parameters like the buffer’s salt concentration or pH may result in the activity of either protein.

Again, neither E. coli with the pBAD/ctpF insert nor those with the pBAD/ctpJ insert showed increased metal resistance to the metals tested.

Experimental process: Some of the experiments themselves required altering to optimize the amount of data obtained from them. For example, the dot blot for expression initially had very faint dots. This was most likely due to low expression levels as indicated by the fact that lanes 1 and 3 in figure 9, which are solubilized membrane samples, show little target protein compared to total protein content. In order to rectify the faint dots in the dot blots, longer exposure times were used during the Bio-Rad Gel/Chemi Doc capture.
Another experiment that had to be altered was the metal resistance experiment. When testing for potassium, the metal inhibition halo experiment became impractical due to potassium’s low toxicity. The drop test experiment was developed to address this issue by allowing for the bacteria to be exposed to higher concentrations of potassium. Only the TKW3205 and TRK2000 strains were used in the drop test since those strains are more sensitive to potassium.

*Issues during experiments:* During the solubilization optimization experiments, a marked drop in % yield can be observed between the first set of experiments (table 1) and the second set of experiments (table 2). This is probably due to the fact that the second set of experiments only had 1 mg of protein per condition compared to 9 mg of protein per condition and thus any loss of protein (due to pipetting error, etc.) was amplified in % lost.
Bibliography