N-Terminal Domains of Copper ATPases In Plasmodium Falciparum, Dictyostelium Dicsoideum, Human ATP7B and a Chimera of Atox1 with Metal Binding Domain 4 of ATP7B

Javan Kilango Kisaka
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N-TERMINAL DOMAINS OF COPPER ATPASES IN PLASMODIUM FALCIPARUM, DICTYOSTELIUM DICSOIDEUM, HUMAN ATP7B AND A CHIMERA OF ATOX1 WITH METAL BINDING DOMAIN 4FOUR OF ATP7B

by

Javan Kilango Kisaka

A dissertation submitted to the Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy Chemistry Western Michigan University June 2016

Doctoral Committee:

David Huffman, PhD., Chair
Blair Szymczyna, PhD
Gellert Mezei, PhD
Pamela Hoppe, PhD
Copper transporting ATPases (Cu-ATPases) are essential for mediating copper uptake, maintaining cellular copper levels and prevention of accumulation of toxic copper. The most studied copper ATPases are the Menkes and Wilson proteins in eukaryotes, CopA and CopB found in bacteria. The precise interaction of the metal binding domains with their copper donor, the association of each metal binding domain within the N-terminal portion of the protein, how they communicate with each other, and the eventual metal ion translocation across the membrane, is little known. The variation in number of the cytosolic metal binding domains may also offer clues on the structural and functional roles these Cu-ATPases play in organisms. In order to gain more insight on the structural organizations of copper ATPases, I have studied the N-terminal domains of three copper ATPases: (1) Wilson disease protein, (2) Dictyostelium discoideum Copper ATPases and (3), Plasmodium falciparum copper ATPase (PfCuP-ATPase).

The N-terminal portion of Wilson protein (ATP7B) is made of six metal binding domains which can receive copper(I) from a copper donor, HAH1, that forms a stable adduct with metal binding domain four. I engineered a chimera of this metal binding
domain with the copper chaperone HAH1. When the chimera was denatured using guanidine hydrochloride, it unfolded in three phases. The first and second midpoint of unfolding is 1.37 M and 5.6 M GuHCl respectively. The midpoint of thermal unfolding is 78 °C. The chimera was crystalized by hanging drop vapor diffusion at room temperature using Hampton crystallization screen.

Dictyostelium discoideum has three copper ATPases: ATP1, ATP7A and ATP3. The number and structural basis of the metal binding domains of these heavy metal transporters is little studied. Using bioinformatics tools, circular dichroism and solution NMR, I have identified two N-terminal domains of ATP1. Domain1 has MXCXXXC metal binding motif that is uncommon of PIB type Cu ATPases. Copper binding studies reveal that this domain has a high affinity for copper(I), with a $K_D$ of $2.2 \times 10^{-18}$ M.

PfCuP-ATPase is a copper ATPase that is involved in copper metabolism in Plasmodium falciparum. The number of metal-binding domains and their functions is only partially understood. I have identified three N-terminal domains of PfCuP-ATPase and pursued biophysical studies to better understand the structural organization of these domains. Domain 3 is the most soluble and relatively resistant to chemical denaturation with guanidine hydrochloride. $^{15}$N-labelled samples were prepared and $^1$H-$^{15}$N HSQC NMR experiments were performed for four different constructs. NMR resonance assignments and subsequent Rosetta modelling shows domain 3 to have a ferredoxin fold that is similar to the metal binding domains of human Wilson disease protein. Copper binding studies were performed under anaerobic conditions with $\text{Cu}^+$(CH$_3$CN)$_4$ as the copper source, and we obtained a $K_D$ of $1.35 \times 10^{18}$ M. The presence of a two domain construct, like that of human Wilson protein metal-binding domains five and six, yet
lacking a metal-binding motif in domain three is intriguing. This finding highlights the structural versatility and stability of the dual ferredoxin fold.
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I am deeply indebted to the people whose support made it possible for me to complete this degree.

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My family has been key to all my success. I am blessed with unwavering support from my parents, brothers, sisters, in-laws, grandparents, nephews and nieces. Your love has surpassed the distance that separates us.

I am dedicating this dissertation to my mom and dad, Mr. and Mrs. Susan Kisaka. Your love is beyond measure. To God be the glory.

Javan Kilango Kisaka
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<td>Atox1</td>
<td>Antioxidant 1 copper chaperone</td>
</tr>
<tr>
<td>ATP7A</td>
<td>Menkes Disease-associated protein</td>
</tr>
<tr>
<td>ATP7B</td>
<td>Wilson Disease-associated Protein</td>
</tr>
<tr>
<td>ATP1</td>
<td><em>Dictyostelium discoideum</em> copper transport ATPase 1</td>
</tr>
<tr>
<td>ATP3</td>
<td><em>Dictyostelium discoideum</em> copper transport ATPase 3</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid Assay</td>
</tr>
<tr>
<td>BCS</td>
<td>Bathocuproinedisulfonic acid</td>
</tr>
<tr>
<td>CCS</td>
<td>Copper Chaperone for Superoxide Dismutase</td>
</tr>
<tr>
<td>Cco</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
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<td>COX11</td>
<td>Cytochrome c Oxidase Copper Chaperone</td>
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<td>CoPA</td>
<td><em>E. coli</em> Copper Transporting P-type ATPase</td>
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<tr>
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<td>Cysteine Proline Cysteine</td>
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<td>CP</td>
<td>Serum Ceruloplasmin</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GuHCl</td>
<td>Guanidine Hydrochloride</td>
</tr>
<tr>
<td>HAH1 (or Atox1)</td>
<td>Human ATX1 Homologue</td>
</tr>
<tr>
<td>hCTR1</td>
<td>Human Copper Transporter 1</td>
</tr>
<tr>
<td>hCP</td>
<td>Human Ceruloplasmin</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LIC</td>
<td>Ligase Independent Cloning</td>
</tr>
<tr>
<td>MP</td>
<td>Membrane Protein</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PfCuP-ATPase</td>
<td>Plasmodium falciparum Copper ATPase</td>
</tr>
<tr>
<td>PfCuP-D1</td>
<td>Plasmodium falciparum Copper ATPase Domain 1</td>
</tr>
<tr>
<td>PfCuP-D2</td>
<td>Plasmodium falciparum Copper ATPase Domain 2</td>
</tr>
<tr>
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<td>Plasmodium falciparum Copper ATPase Domain 3</td>
</tr>
<tr>
<td>PfCuP-D2-3</td>
<td>Plasmodium falciparum Copper ATPase Domain 2-3</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDSPAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
</tbody>
</table>
List of Abbreviations – Continued

TEV ................................................................. Tobacco Etch Virus
TGN ........................................................................... Trans-Golgi Network
TRX ............................................................................. Thioredoxin
WD.............................................................................. Wilson disease
WDP ............................................................................. Wilson disease protein
WD4 .......................................................... Wilson disease protein metal binding domain 4
WD5-6 .................................................. Wilson disease protein metal binding domains 5 and 6
ZntA.......................................................... Zinc ATPase
CHAPTER 1

COPPER METABOLISM

1.1 Introduction

A common thread running through the tapestry of aerobic life is the strict requirement for first row transition metal ions, such as manganese, iron, cobalt, nickel, copper, and zinc.\(^1\) The chemistry catalyzed by these metals is diverse, ranging from hydrolytic chemistry all the way to C-H activation.\(^2\) The critical nature of these metals is realized even the act of respiration,\(^3\) wherein molecular oxygen is poised strategically between an iron and a copper atom, prior to its stepwise reduction to water.\(^4\) Interestingly, copper is required for high affinity iron uptake,\(^5\) in that copper enzymes modulate the oxidation of iron(II) to iron(III),\(^6\) during its transport process – a step necessary to abrogate the formation of rust (iron(III)oxide).\(^7\) As a side note, the most eukaryotes store iron, in the form of rust, within the nanosized protein ferritin.\(^8\)

Prior to 1995, the uptake and delivery of these first row transitions metals to their physiological targets was poorly understood.\(^9\) One critical feature for copper is the requirement for a pumping mechanism for mobilization within cells or for movement out of cells.\(^10\) The concentration of ‘free copper’ within cells is quite low,\(^11\) although this depends on the exact definition of ‘free’ and it also depends on the state of the system. In some cases, rapid mobilization of transition metals ions occurs.\(^12\) A very good example of the latter occurs during embryonic development, with the formation of zinc
‘sparks’,\textsuperscript{13} as the zinc moves to the proper location of the cell. In seeds and plants, mobilization of transition metals can be tracked via X-ray fluorescence.\textsuperscript{14} Since copper is so vital, understanding how it is transported and delivered within cells advances knowledge of both nutrition and disease.\textsuperscript{15} The interconnectedness living things means that ‘simpler’ organizations affect ‘more complex’ organisms.\textsuperscript{16} In fact, humans do not dominate the world, from a genetic diversity standpoint.\textsuperscript{17} The requirement for copper in all organisms and the need to elucidate the cellular chemistry of copper, has stimulated our laboratory to cross phylogenetic boundaries, broadly speaking. The goal of this dissertation is to address key features of the chemistry of copper that impact world health and well-being.

Copper is an essential micronutrient as it plays important catalytic and structural roles in many enzymes. The ability of copper to cycle between oxidized and reduced states is harnessed by Cuproenzymes. This property is required in many redox reactions. However, the reduced copper (Cu(I)) is potentially toxic to cells since it can lead to Fenton-like chemistry.\textsuperscript{18} The metabolism of copper in cells must therefore be tightly controlled. Copper homeostasis involves the uptake, distribution, and secretion to meet essential cellular requirements. It is crucial to minimize the amount of free Cu(I) in cells. The dietary intake of copper usually exceeds tissue demands. The homeostatic mechanisms must therefore modulate uptake and facilitate export through the bile. Copper uptake in cells is mediated by a copper permease protein (Ctr1).\textsuperscript{19}

Intracellular copper is distributed by copper chaperones to different pathways and organelles. This includes the copper chaperone for superoxide dismutase (CCS) that distributes copper to superoxide dismutase (Cu/Zn SOD) in the cytosol and mitochondria,
Antioxidant protein 1 (Atox1) may transfer copper to the nucleus\textsuperscript{20} and secretory pathways.\textsuperscript{21} The excretion of excess copper is mediated by copper efflux ATPases, ATP7A and ATP7B. These may also pump Cu to the Golgi apparatus. The trafficking of copper pathways in the cell is illustrated in Figure 1. Copper is required in many enzymes where it functions as an essential cofactor.\textsuperscript{22,23} It is also required for cellular processes such as oxidative phosphorylation, iron metabolism, pigment formation, connective tissue cross-linking, catecholamine synthesis, and antioxidant defense.\textsuperscript{24}

Due to these distinct cellular functions, copper deficiency has profound effects that lead to neurodegeneration and many other disability conditions.\textsuperscript{25} On the other hand, excess copper levels have adverse effects. The impairment in biliary copper excretion results in the liver, brain, and ocular copper overload associated with Wilson's disease.\textsuperscript{26} The redox properties of copper may generate hydroxyl radicals through the Fenton reaction.\textsuperscript{27} Therefore, copper levels are tightly regulated to maintain sufficient supply and minimize toxic effects.\textsuperscript{28}
Figure 1.1
Copper trafficking pathways showing the movement of copper as traced from the small intestines to various targets.
1.2 Copper import transporters and metallochaperones

1.2.1 CTR1 and CTR2
High affinity copper uptake is facilitated by a copper transport protein (Ctr1) which is located both in the plasma membrane and the intracellular vesicles. Ctr1 retrieves copper from circulation and from carrier proteins, and transports it across the basolateral plasma membrane of the kidney, liver, placenta and mammary glands. Ctr1 makes dietary copper available for further utilization by facilitating absorption and its release from the vesicles. Copper can also enter the cells via a low affinity copper transporter, Ctr2 that may be involved in the release of copper from the lysosomes or endocytic vesicles. Ctr2 is a homolog of Ctr1 that may be involved in copper import and intracellular homeostasis. The overexpression of Ctr2 has been associated with increased copper uptake. Ctr1 and Ctr2 donate Cu (I) to metallochaperones that distributes the metal ion to various pathways. Copper metallochaperones are proteins that function as intracellular Cu(I) ion shuttles, distributing Cu(I) ions to specific partner proteins, thereby overcoming a high copper chelation capacity of the cytoplasm. In the cytoplasm of many cells, metallothioneins and glutathione molecules buffer Cu(I) to deplete any free copper ion.

1.2.2 Atox1
Atox1 was first discovered in yeast, but also present in mammals. Human Atox1, also referred to as HAH1, is a soluble protein that delivers copper to the Menkes (ATP7A) and Wilson disease protein (ATP7B). ATP7A and ATP7B are integral membrane proteins that transport copper in the trans-Golgi network (TGN) or across the plasma membrane. Both ATP7A and ATP7B have six N-terminal metal binding domains that
have similar structure to HAH1. HAH1 delivers copper to any of the six metal binding domains. HAH1 has a $\beta\alpha\beta\alpha\beta$ ferredoxin fold with the metal heavy metal binding motif, MTCXXC, located at the end of the loop of the joining the first $\beta$ sheet and $\alpha$ helix (Figure 1.2).\(^{37}\)

![Figure 1.2 Crystal structure of HAH1 showing Cu(I) bound to cysteine residues in the CXXC copper binding motif. PDB: 1FEE](image)

One study suggests that HAH1 first delivers copper to domain two, as the first point of entry.\(^{38}\) The delivery of copper from HAH1 to the metal binding domain of ATP7B may be facilitated by protein-protein interactions for the direct transfer of copper ions.\(^{38}\) The binding of copper to the metal binding domain of WDP induces conformational rearrangements. The protein-protein interaction may be due to the variations in electrostatic metal binding domains.\(^{39}\) HAH1 plays an important role in the copper metalation pathways as well as intracellular copper efflux.\(^{34}\) It may also function to
protect neurons from oxidative stress. Overexpression of Atox1 can increase neuronal viability under stress conditions, such as serum oxidation and deficiency.\textsuperscript{40} Cells that lack Atox1 have impaired movement of ATP7A in response to copper levels.\textsuperscript{41}

1.2.3 Cox17, Cox11 and Sco1

Cox17 and Cox11 are mitochondrial metallochaperones that deliver copper to cytochrome c oxidase. Unlike Atox1 which delivers copper directly to the target protein, Cox17 donates Cu(I) to cytochrome c oxidase (CcO)\textsuperscript{42} by involving two accessory factors Cox11 and Sco1. Cox17 is found within mitochondrial intermembrane space (IMS) and acts as a copper donor to both Sco1 and Cox11. \textsuperscript{43} Cox17 contains conserved cysteines that makes the protein to have up to three different oxidation states in the IMS. A fully oxidized Cox17 has three disulfide bonds, partially oxidized Cox17 has two disulfide bonds while a fully reduced Cox17 is without any disulfide bond.\textsuperscript{44,45} The fully oxidized state of Cox17 has no free thiols and is not able to bind copper, the partially oxidized form of Cox17 can bind one copper ion because the other cysteine residues are involved in disulfide bonds.\textsuperscript{44,46}

Cox17 is a protein made of 69 amino acid residues. The N-terminal portion has about 20 residues that forms unstructured segment followed by two $\alpha$-helical hairpin domain (Figure 1.3).\textsuperscript{22,46,47}
The molten globular state of Cox17 is fully reduced and binds four Cu(I) ions with the six cysteine residues.\textsuperscript{44,48}

In addition to Cox17, three other proteins, Cox11, Cox19 and Sco1 mediate the delivery of copper to cytochrome c oxidase-Cco (Figure 1.1). Cox 7 was the first protein to be identified in copper ion delivery to Cco.\textsuperscript{42} The functional importance of Cox17 is evidenced in yeast where mutants of Cox17 are deficient in respiratory growth and Cco activity.\textsuperscript{42} Sco1 is an integral inner membrane protein with a single transmembrane helix and a globular domain projecting into the IMS.\textsuperscript{49} Cells with null Sco1 have diminished Cco activity and are deficient in respiration.

Cox11 is found both in eukaryotes and in gram-negative bacteria. Cox11 has a highly soluble domain that has an N-terminal fragment anchoring the protein to the membrane.
Banci et al. solved the solution structure of the Cox11 from *Sinorhizobium meliloti*. The apo form has an immunoglobulin-like fold which is β-barrel structure.\textsuperscript{50} The importance of Cox11 was first demonstrated by Tzagoloff. Yeast lacking Cox11 have no CcO activity and are deficient in heme \textit{a}.\textsuperscript{51} Hiser et al. have demonstrated that Cox11 may function primarily in the formation of the Cu\textsubscript{B} site of Cco.\textsuperscript{52} Cox11 may also function as a co-chaperone that facilitates the delivery of copper to Cco through another molecule. Yeast Cox11 is a 34-kDa protein that is anchored by a single transmembrane domain to the inner mitochondrial membrane. It is dimeric both in the apo and Cu(I) bound form.\textsuperscript{53}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{cox11.png}
\caption{Solution structure of Cox11}
\end{figure}

It binds one copper ion per monomer with three conserved cysteine residues.\textsuperscript{53}

\subsection{1.2.4 CCS1}

Ccs1 is a copper metallochaperone for Cu, Zn-superoxide dismutase. Ccs1 is made of three domains. Domains 1 and 3 bind Cu(I) while domain 2 interacts with Sod1.\textsuperscript{54,55} Ccs1 is present in many organisms. Studies have shown that Ccs1 is required for activation of
Sod1 in yeast, Drosophila melanogaster, and mice. Binding of Cu(I) to Ccs1 induces activation of the reduced monomeric Sod1 and facilitates the formation of a disulfide bond between Cys57 and Cys146. The stability of this disulfide bond is postulated to be due to both the low solvent accessibility of Cys146 and the dimerization of Sod1. Upon reduction of the disulfide and in the absence of the metal cofactors, the Sod1 dimer is destabilized and exists as an inactive monomer. Ccs1 has three domains. The N-terminal domain has a βαβαβαβ fold, similar to Atx1, and is capable of binding one Cu(I) ion. Domain 1 of yeast Ccs1 is not essential for normal growth, but is required for the activation of Sod1. Domain 2 has an eight-stranded β-barrel structure, analogous to Sod1. In humans, domain 2 of Ccs1 has a Zn(II) binding site, but lacks the ligand for copper coordination. In yeast, domain 2 of Ccs1 lacks both the Zn and Cu binding sites. Domain 2 of Ccs1 is required for docking during the activation of Sod1. Domain 3 is found in the short segment of C-terminal. This domain contains a CXC motif for metal binding, disulfide formation or activation of Sod1.

1.2.5 Serum Ceruloplasmin

Human serum ceruloplasmin (hCp) is a copper-containing glycoprotein that belongs to a family of multi-nuclear “blue” copper oxidases. Cp also called iron ferroxidase and it is involved in iron metabolism. It was first isolated in 1948. It has a single polypeptide chain of 1046 amino acid residues. A glycosylphosphatidylinositol (GPI)-anchored form of Cp is expressed in the mammalian central nervous system (CNS), while the secreted form is expressed by the liver released in serum. The GPI-Cp plays a role in iron homeostasis and antioxidant defense, where it converts the toxic ferrous iron into the
nontoxic ferric form\textsuperscript{68,69} CP belongs to a multicopper oxidase family of enzymes that is characterized by the presence of three types of spectroscopically distinct copper sites.\textsuperscript{70} The type I copper sites have charge transfer between the sulfur ligand of cysteine and the Cu(II) center. Type II copper sites have copper coordinated to four imidazole nitrogens. The type II and type III copper sites form a trinuclear copper cluster at the interface of domain one and six.\textsuperscript{71} In addition to the three copper ions in the trinuclear cluster, three coppers are found in three mononuclear sites in domain 2, 4 and 6. In each of the mononuclear sites two histidine residues and a cysteine coordinate the copper ion with an additional methionine ligand in domain 4 and 6.\textsuperscript{6} Cp has four known functions. It plays a role in copper transport, and ferroxidase and amine oxidase activity, and also functions as an antioxidant by preventing the formation of free radicals in the serum.
Although ceruloplasmin requires copper for its function, the mechanism of the secretory compartment by which copper is incorporated into newly synthesized ceruloplasmin is unknown. When copper is not incorporated it leads to production of apo-ceruloplasmin, which has a short half-life of about five hours in the blood.\textsuperscript{72, 73}

1.2.6 P-type ATPases

P-type ATPases are integral membrane proteins that use ATP energy to maintain metal ion homeostasis, electrochemical gradients and lipid bilayer asymmetry in cells.\textsuperscript{74, 75}
There are 11 classes of the P-type ATPase superfamily.\textsuperscript{76} Class IB (PIB) is the largest and found in many prokaryotic and eukaryotic organisms.\textsuperscript{77,78} They are essential for cellular regulation of heavy metals such as Co(II), Zn(II) and Cu(I).\textsuperscript{79} Due to their toxic nature, these metals are tightly regulated and have extremely low intracellular concentrations. Cu(I) transporting ATPases are the most prevalent P\textsubscript{IB} ATPases.\textsuperscript{79} They are required for the regulation of copper metabolism in many organisms. In humans there are two Cu(I)-transporting P\textsubscript{IB} ATPases, ATP7A and ATP7B. Defects or mutation in these proteins cause Menkes and Wilson’s disease, respectively.\textsuperscript{80} Their up-regulation may cause resistance to cancer chemotherapy and Alzheimer’s disease.\textsuperscript{81,82}

\textbf{1.2.6.1 Copper efflux transporters: Menkes protein, Wilson disease protein, PfCuP-ATPase}

Wilson disease protein (ATP7B) transports copper into the secretory pathway for subsequent incorporation into ceruloplasmin and excretion into bile.\textsuperscript{83} When there is an increase of copper concentration in the hepatocytes, the Wilson disease protein translocates from the trans Golgi network (TGN) to sequester excess copper in cytoplasmic vesicular compartment near the membrane, and copper is excreted into the bile.\textsuperscript{84} When copper concentration decreases, ATP7B returns to the TGN. The copper dependent movement of ATP7B provides a sensitive mechanism of copper homeostasis by hepatocytes. Mutations in the ATP7B gives rise to impaired biliary copper excretion and decreased serum ceruloplasmin.\textsuperscript{85}

The proteins associated with Menkes (ATP7A) and Wilson (ATP7B) diseases are integral membrane proteins of the P\textsubscript{IB} ATPases that couple ATP hydrolysis with transport of copper (I) ions across the membranes.\textsuperscript{86,80,87,88} Mutation in ATP7A and ATP7B cause
genetic disorders of copper metabolism leading to copper starvation or toxicity, respectively. Both the ATP7A and ATP7B have similar structural features. Their N terminal region is composed of six metal binding domains (MBDs). These MBDs interact with and are able to receive copper ions from the copper chaperone HAH1.

1.2.6.2 Menkes disease protein

ATP7A is an X-linked inherited neurodegenerative childhood disorder caused by the absence or dysfunction of a P-type ATPase (ATP7A) encoded on the X chromosome. It delivers copper to the secretory pathway of eukaryotic cells. ATP7A is located in the chromosome at Xq13.2-13.3 and has 23 exons that span approximately 150 kb. The ATP7A gene is expressed in all tissues except the liver. Mutations in the ATP7A gene results in poor distribution of copper to the body's cells. The result is accumulation of copper in some tissues, such as the small intestine and kidneys, while the brain and other tissues have unusually low levels of copper. Low levels of copper may reduce the activity of many copper-containing enzymes that are required for the structure and function of bone, skin, hair, blood vessels, and the nervous system. Mutations that impair the function of ATP7A gives rise to Menkes syndrome, which is characterized by sparse, kinky hair, loss of weight and deterioration of the nervous system.

1.2.6.3 Wilson disease protein (ATP7B)

ATP7B is a genetic disorder of copper homeostasis that is characterized by the excessive accumulation of copper metal in the liver. ATP7B is a copper transporting P-type ATPase that is predominantly expressed in the liver, that results from mutations in the
ATP7B gene.\textsuperscript{93} ATP7B has eight transmembrane domains that form a channel through which copper ions pass across the membrane. In hepatocytes, the copper is transferred to the ferroxidase ceruloplasmin and secreted into the serum.\textsuperscript{93} Subcellular localization of ATP7B varies according to changes in the copper concentrations. At low copper levels, the protein is localized at the Golgi compartment where it is believed that copper loading of ceruloplasmin takes place.\textsuperscript{94,85} When copper levels increase, ATP7B translocates to cytoplasmic dispersed vesicles.\textsuperscript{85,95,96} The vesicles transport copper to the bile for eventual excretion.\textsuperscript{97,98}

ATP7B has characteristic features of P-type ATPases. The N-terminal domain has six heavy metal binding domains (MBDs) with the MXCXXC motif, which form the metal binding site.\textsuperscript{99} It also has eight transmembrane domains with a CPC motif within the sixth membrane domain, which facilitates the translocation of the cation through the membrane.\textsuperscript{100,101}

In addition to the heavy metal binding domains, ATP7B has other cytosolic domains. The ATP binding domain is subdivided into two subdomains: the phosphorylation domain (P-domain containing the DKTGT motif)\textsuperscript{102} and the nucleotide-binding domain (N-domain containing the GDGIND motif).\textsuperscript{103} The actuator domain (A-domain) located between the fourth and fifth transmembrane helices, initiates the phosphorylation process of ATP7B. The topology of N-domain is $\beta a a \beta \beta a a \beta \beta$ consisting of a central six-stranded anti-parallel $\beta$-sheet and two $\alpha$-helical hairpins, one on each side of the $\beta$-sheet.\textsuperscript{104}
Each of the N-terminal metal binding domains has a ferredoxin fold and are connected by linkers between them. Figure 1.6 shows a double ferredoxin fold of domains 5 and 6 of ATP7B.

The number of N-terminal domains of varies between species. For instance, in yeast, the ATP7B homologue known as Ccc2 has only two metal binding domains and can bind maximum of two copper ions. The *Caenorhabditis elegans* homolog contains three metal binding domains, while rat has 6 domains, with one lacking the MXCXXC motif.
1.2.7 The transfer of copper from the HAH1 chaperone to the metal binding domains of ATP7B

HAH1 is a copper chaperone that delivers copper ions to the metal binding domains of ATP7B. It has 68 amino acids residues and a molecular weight of 7.5 kDa. Its structure is a ferredoxin fold (βαββαβ) and has a MTCGGC motif similar to the metal binding domains of ATP7B. Each of the metal binding domains of ATP7B also has about 70 amino acids and a similar sequence and structure. Each domain folds independently and can bind one equivalent of copper (I) ion.\textsuperscript{99,109,105,22} It has been proposed that the N-terminal domains of ATP7B play an important role in the tuning of ATP7B activity and modulating the intracellular trafficking rates of the ATP7B.\textsuperscript{98,110,111}

The transfer of Cu(I) from the chaperone to the MBDs of ATP7B is mediated by a metal-bridged intermediate.\textsuperscript{112} In one study, the binding affinity ($K_{Cu}$) of the six MBDs of ATP7B was found to be higher than the $K_{Cu}$ of the metallochaperone HAH1.\textsuperscript{113} The copper ion may undergo inter-domain transfer until it is eventually transported across the membrane. The copper ion is then transferred to acceptor protein, ceruloplasmin in the lumen (Figure 1.28). The transfer of copper(I) to the metal binding domains of ATP7B has been studied using various methods, such as NMR and metal transfer assays. Achila and co-workers\textsuperscript{105} used a construct containing the last two metal binding domains(MBD5-6) to study the copper transfer from HAH1 to these domains. Titration of Cu(I)-HAH1 to the apo MBD5-6 construct revealed that HAH1 does not transfer copper to domains 5 and 6. There was no copper exchange or complex formation observed via NMR. Interestingly, this construct was able to receive copper from Cu(I)-
MBD4 when MBD5-6 was titrated with holo MBD4. It was observed that domain 6 received copper before domain 5, without any complex formation with HAH1.

Figure 1.7. Movement of copper ions across the TGN membrane

Domain 4 also forms an adduct with Cu(I)-HAH, and to a lesser extent with MBD2. Banci and co-workers\textsuperscript{22} used solution NMR to study the interaction of Cu(I)-HAH1 with MBD3-4 of ATP7B. When MBD3-4 was titrated with Cu(I)-HAH1, HAH1 formed a complex with MBD4, MBD3 also did not acquire copper from HAH1. These
observations were in contrast with the interaction of MBD1-6 with Cu(I) HAH1. Each domain in this construct was able to receive copper from Cu(I)-HAH1. However, domains 1, 2 and 4 were able to form complexes of a copper(I)-bridged adduct with HAH1 while domains 3, 5 and 6 received the copper metal from the metallochaperone without accumulation of the protein-protein adduct.

A separate study by Fatemi and co-workers used NMR spectroscopy to probe the structural and dynamic properties of MBD4–6 and MBD1-6 but their results were not in agreement studies of Banci et al. They determined that the three domains in the MBD4-6 construct have independent mobility in solution. Domain 4 tumbles independently of domain 5 and 6. This may be due to the fact that domain 4 is separated by a long linker from domain 5 while domains 5 and 6 have a shorter linker between them, which limits their interdomain movements. In this study it was found that domains 4, 5, and 6 tumbled independently of each other both in the apo state and Cu(I)-bound state. It was shown that the construct MBD4-6 interacted with the metallochaperone both in the apo and holo forms. Domain 4 appeared to have more interaction with HAH1, suggesting that the transfer of copper from HAH1 to this domain is driven by protein-protein interactions that occur even in the absence of copper. This may be plausible because domain 4 has a net negative charge at pH 7 with a pI of 3.81 while HAH1 has a pI of 7.5 and a net positive charge (theoretical values). The flexible linkers between individual MBDs of ATP7B suggest that each domain may receive copper from HAH1 independently, with domain 4 being the most likely to form a complex with Cu(I) bound HAH1. The absence of domain four in mouse ATP7B questions the hypothesis that HAH1 first transfers copper to MBD4 and that domain 4 subsequently transfers copper to other MBDs. Since
both HAH1 and MBDs of ATP7B have a similar structure ($\beta\alpha\beta\alpha\beta$ ferredoxin folds), the presence of complementary charges at the surface of these proteins suggest that HAH1 forms adducts and transfer the Cu(I) metal ion to the MBDs through ligand exchange.\textsuperscript{109}

1.2.8 The Wilson disease

Wilson’s disease (WD) is caused by accumulation of copper in major organs due to a defect in ATP7B. Copper deposition in the cornea results in the Kayser-Fleischer (KF) ring, a rusty brown ring around the cornea of the eye. There are nearly 500 identified mutations in ATP7B, and 300 of these mutations have been associated with Wilson disease.\textsuperscript{116} The mutation may be at any position of the gene. The most common type of mutation found in ATP7B is a missense mutation. Genetic mutation of ATP7B gene in patients with WD vary across various races and geographic regions.\textsuperscript{117} H1069Q mutation is the most common in WD patients from European countries such as Sweden, Italy and Romania.\textsuperscript{118} R778L is most prevalent mutation in East Asia.\textsuperscript{119}

1.3 Copper metabolism in \textit{Plasmodium falciparum}

1.3.1 Introduction

Understanding copper metabolic pathways in \textit{Plasmodium falciparum} will be helpful in discovering possible copper chelators that may be used in new antimalarial therapy development. Copper is an essential nutrient that \textit{Plasmodium falciparum} needs to colonize its host.\textsuperscript{120} The cellular copper levels are carefully regulated by a copper efflux
protein (PfCuP-ATPase) to prevent its toxic levels. The work of Asahi et al. shows that chelation of Cu(I) inhibits growth of *Plasmodium falciparum* at the ring stage.

### 1.3.2 Malaria: malarial parasites, current treatment and drug resistance

Malaria has remained a major threat to the public health and economic development, especially in tropical regions of the world. The majority of the world’s population lives in regions where malaria is common. Human malaria is caused by *Plasmodium* parasites transmitted by the *Anopheles* mosquito. There are four plasmodium parasites that cause malaria: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. Of the four parasites, *Plasmodium falciparum* is the most lethal.

Spread of malaria is largely due to the resistance to anti-malarial drugs and insecticides, lack of proper public infrastructure, movement of population, and environmental changes.

Presently, due to the lack of an effective malaria vaccine, control and management of *Plasmodium falciparum* based malaria is mediated by antimalarial drugs. This has led to the spread of drug-resistant *Plasmodium falciparum* parasites continue to threaten global malaria control and elimination programs. In 2002, it was estimated that approximately 2.2 billion people were exposed to *Plasmodium falciparum* malaria, with 300-600 million reported clinical cases. Of these reported cases, 70% were in Africa, while 25% were in South East Asia. Malaria has an annual mortality rate of about a million. In 2010 3.3 billion people were at risk of acquiring a malaria infection,
216 million cases of malaria were reported worldwide and 655,000 of the infected died (WHO Malaria report, 2011). In 2013 there were an estimated 198 million cases and 584,000 deaths reported (WHO Malaria report, 2014). It is one of the most infectious diseases mankind has experienced. Malaria infection is predominantly caused by *Plasmodium falciparum*.

In many cases the parasite has developed resistance to currently available anti-malarial drugs. The mechanism of action of some of these drugs, such as artemisin, deferoxamine and quinolone, is their ability to interact with metal ions and creating free radical intermediates. These metal chelators are believed to inhibit parasite growth though radical formation. Resistance of malarial parasites to some of the existing drugs requires the need to develop new therapeutic agents. Integral membrane proteins which are metal transporters are increasingly becoming targets for potential drug development. These proteins are essential for metabolism of metal ions and are required for the growth and replication of *Plasmodium* parasites. Understanding structure and function of these proteins will provide valuable information that may be applied in drug development.

**1.3.3 Growth and development of *Plasmodium falciparum***

Understanding the *Plasmodium falciparum* stages of growth and development will be valuable in exploring new ways of interfering with the parasites life cycle. Malaria parasites have a complex life cycle in their two host organisms: humans and female *Anopheles* mosquitoes. The life cycle of malarial parasites is one of the most complicated and fascinating areas of study in cell biology, molecular biology, and
immunology of any living organism. The intricate biology of *Plasmodium falciparum* has proved to be challenging for researchers for decades and has been a major setback for the development of a malarial vaccine. As a result, malaria has continued to be a devastating disease in developing countries, especially in sub-Saharan Africa. Two of the major challenges in understanding the parasite’s life cycle is its abilities to change its cellular and molecular makeup, which is controlled by a genome with more than 5000 genes, and it is interaction with the mammalian host and the mosquito vector.\(^{134}\) The complex development of malarial parasites may explain why the mode of transmission of malaria was unknown until the end of nineteenth century.

Initially, in 1903, it was erroneously observed that the sporozoite stage of malarial parasites directly infects the erythrocytes. This mistake hindered the progress of studies on the initial stages of the malarial infection in the human host. Four decades later Shortt & Garnham\(^ {135}\) demonstrated that the sporozoites first infects the hepatocytes, and then the erythrocytes. Much success in understanding the pre-erythrocytic stages of malarial parasites has been achieved through advancement of gene manipulation technologies and *in vivo* imaging.\(^ {135, 136}\) Valuable data has now been collected about the various molecular and cellular mechanisms of sporozoites infection of the liver. The genes that are essential for the development of the malarial parasites in the liver are being targeted for the development of potential malarial vaccine.\(^ {137}\)

In humans, the *Plasmodium falciparum* parasite’s life cycle begins in the liver, with subsequent infections of the red blood cells. Parasites in the blood are responsible in causing malarial symptoms. Parasites, in the form of sporozoites, are injected into the blood stream by a female *Anopheles* mosquito while it feeds on human blood.\(^ {138}\) These
sporozoites then travel to the liver where they invade liver cells. In the hepatocytes the sporozoites grow, divide, and release thousands of haploid forms, called merozoites. The merozoites exit the liver cells, re-enter the bloodstream, and invade the red blood cells. In the red blood cells, they multiply asexually and release newly formed merozoites. Thousands of parasite-infected cells in the host bloodstream cause the malarial illness. It is believed that during red blood cell invasion *Plasmodium falciparum* merozoites use multiple receptor–ligand interactions to coordinate a series of events.

Some of the merozoites develop into the asexual form of the parasite that has male and female gametocytes. The gametocytes are ingested by a mosquito when it bites an infected human, and they develop into gametes while in the gut of the mosquito. The male and female gametes fuse together to form diploid zygotes, which develop into ookinetes and eventually oocytes. The oocytes grow and divide into form sporozoites. When the oocytes burst, it releases the sporozoites, which reside in the salivary glands of the mosquito. When it takes a blood meal from a human, the sporozoites are injected into the human blood stream and the cycle re-starts. The host’s fight against *Plasmodium falciparum* has been hampered by the complexity of the life cycle of the parasite. The short-lived extracellular appearance of the merozoites and the intracellular nature of the other asexual forms makes it extremely difficult for the host to mount an effective immune response.

1.3.4 Targeting malarial parasites’ proteins for anti-malarial drugs development

The first genome sequence for the *Plasmodium falciparum* was published in 1998. With the estimated 5,300 genes, researchers around the world aim develop new
antimalarial drugs, vaccines and diagnostic tests. By 2002 the *P. falciparum*, *Anopheles gambiae* and *Homo sapiens* genome sequences were also completed. This was a first milestone in the long search for solutions to the malarial menace. These sequences provide a wealth of information on parasite biology, including the structure, content of the genes, proteins classification and function, and transport metabolism pathways within the parasites.

1.3.5 PfCuP-ATPase: structural organization

*Pf*CuP-ATPase is the largest copper transporter protein with a theoretical molecular weight of 298kDa. The mass is almost double that of *Cryptosporium parvum*, Wilson and Menkes disease protein. The higher mass is due to presence of many inserts between the functional domains.

Unlike other P-type ATPases, *Pf*CuP-ATPase has a single N-terminal copper-binding domain with a MXCXXC motif, which binds copper in reduced state. The protein levels are highest at the trophozoite stage of *Plasmodium falciparum* growth. The protein is found both on the parasite’s membranes and on the surface of infected erythrocytes. This localization suggests that the protein, much like the Wilson and Menkes disease proteins, is involved in the secretion of excess copper ions out of the cell. The Wilson and Menkes disease proteins have six N-terminal domains and are capable of binding up to six equivalents of copper ions. *Pf*CuP-ATPase however, can only bind one equivalent of copper ions due to it having one MXCXXC motif for copper binding. This copper
binding site may individually or in co-operation with other domains within the protein, act as a copper sensor to regulate ATPase transcription in response to copper levels.

*PfCuP*-ATPase is a protein with many puzzling features uncharacteristic of P type ATPases. For instance, the N-terminal metal binding domain has only one MXCXXC motif yet has about 565 amino acids residues. The size of this region is almost equal to the N-terminal domain of Wilson disease protein. Wilson disease protein has six functional metal binding domains in the N-terminal domain. *PfCuP*-ATPase also has 10 predicted transmembrane domains relative to the 8 domains of ATP7B, however, the two additional transmembrane domains have yet to be verified.  

### 1.3.6 *PfCuP*-ATPase expression levels in different stages of *Plasmodium falciparum* life cycle

Rasoloson et al. used an affinity purified MBP-MBD antibody to determine the localization of the *PfCuP*-ATPase. They discovered that *PfCuP*-ATPase is expressed in the trophozoite stage of *Plasmodium falciparum* growth. The protein was not detected in either the uninfected red blood cell or the ring stage. It was determined that protein expression was visible on the surface of infected erythrocytes and on the parasite’s plasma membrane. This result implies that two separate mechanisms are used to transport copper out of the parasite; one involves in the transport of copper ions to the plasma membrane of the parasite and another pathway involves the transport of the metal ion from the parasite plasma membrane to the erythrocyte’s surface and eventual efflux.
A later study by Kenthirapalan et al. revealed the \( Pf\text{-CuP}-\text{ATPase} \) is expressed in all \textit{Plasmodium} life cycle stages and localizes to vesicle-like structures that might represent storage vesicles. The investigators showed through live imaging of the \( Pf\text{-CuP}-\text{ATPase} \) containing parasites that \( Pf\text{-CuP}-\text{ATPase} \) was expressed in both asexual and sexual stages in the blood. The protein also consistently localizes in vesicle-like structures in the parasite’s cytoplasm. The origin of these vesicular like structures remains unknown.

### 1.4 Research objectives

The main research goal is to understand the structural basis of the N-terminal metal domains of copper ATPases by studying four copper proteins in different organisms: Human Wilson disease protein (ATP7B), \textit{Dictyostelium discoideum} copper ATPase (DdATP1), \textit{Dictyostelium discoideum} copper chaperone (DdAtox1), and \textit{Plasmodium falciparum} copper ATPase (\( Pf\text{-CuP}-\text{ATPase} \)).

The cytosolic domains of the metal binding portion, actuator, nucleotide and phosphorylation domains of Wilson disease protein have been studied by NMR. However, the structure of a complete protein has not been determined. There are gaps in the literature on the structure of the copper ATPase and how it couples ATP energy with copper transport. A homologous structure of ATP7B form \textit{Legionella pneumophila} has been solved by X-ray crystallography. Although this structure details the structural organization of ATP7B, the metal binding portion of the protein was missing in the structure. Furthermore, the protein has not been expressed in \textit{E. coli}. Recombinant expression of the ATP7B and the eventual atomic structure determination will help answer key questions of how the enzyme transports copper, how the cytosolic copper is
loaded into the metal binding domains, and the shuttling of the metal ion across the membrane and the effects of mutation on the structure and function of the protein. One of my research goal was to clone the full length Wilson protein by recombinant DNA technology and express the protein in *E. coli* for biophysical characterization. In order to better understand the mode of interaction of the N-terminal domains of ATP7B with its copper donor, HAH1, I engineered a chimera of the fourth metal binding domain four of ATP7B and copper chaperone HAH1 and expressed it in *E. coli*. The aim of this project was to crystallize the chimera in the presence of copper metal with the hope of obtaining a structure that shows the complex formation between the two proteins. The results of this project were expected to shed more light on the mechanism of adduct formation of the copper chaperone HAH1, and metal binding domains of ATP7B.

A second project was undertaken to study the N-terminal domains of *Plasmodium falciparum* copper P type ATPase. Despite copper being found to be essential in the growth and development of *Plasmodium falciparum*, and the discovery of *PfCuP*-ATPase as a copper transport protein involved in the metabolism of the metal ion in *Plasmodium* parasites, the structural organization of the ATPase has not been determined. To better understand its structure and function I pursued biophysical studies of the the N-terminal domains of *PfCuP*-ATPase using bioinformatics analysis, circular dichroism, metal binding studies and solution NMR. The goal of this project was to determine the number of the N-terminal domains, the copper binding affinity of PfCuP-ATPase, and determine the structures of these domains. The findings of this study were expected to highlight the structural organization of the ATPase.
A third project was to define the N-terminal domains of *Dictyostelium discoideum* copper ATPase (*DdATP1*). *Dictyostelium discoideum* is a social amoeba that inhabits forest soils. Its main food source is bacteria. It is speculated that the organism uses copper and zinc as its brass dagger, during feeding, where copper is thought to be involved in bacterial killing in the phagosome of the amoeba. The structure and function of this protein is little known. Using protein bioinformatics tools, two metal binding domains were identified in the N-terminal portion of the copper ATPase. The goal of this project was to clone and express the individual domains in *E. coli* and determine the copper binding affinity of the N-terminal domain of *DdATP1* and compare with that of its copper donor, Atox1 (*DdAtox1*). The results of this project are expected to help us understand the chemistry of phagocytosis.
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CHAPTER 2
MATERIALS AND EXPERIMENTAL METHODS FOR CLONING AND EXPRESSION OF WILSON DISEASE PROTEIN

2.1 Introduction

This chapter describes in details all the reagents, biological supplies, equipment as well as procedures followed for plasmids preparation, protein expression and purification.

2.2 Chemical reagents, plasmids, kits and instrumentation

This section describes the chemical reagents and kits that were used for all experiments. Separate tables contain the list of these materials used.

Table 2.1 Chemical reagents

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<td>13C Glucose</td>
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<td>Cambridge Isotope</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Coomasie Blue R-250</td>
<td>20278</td>
<td>Thermo Scientific</td>
<td></td>
</tr>
<tr>
<td>Dibasic sodium phosphate</td>
<td>S-5136</td>
<td>Sigma Aldrich</td>
<td></td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>1758-9030</td>
<td>Inalco Sas</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>H45476</td>
<td>Mallinckrodt chemicals</td>
<td>99.7%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>G33-1</td>
<td>Fisher Scientific</td>
<td>99.9%</td>
</tr>
</tbody>
</table>
Table 2.1 Chemical reagents – Continued

<table>
<thead>
<tr>
<th>Chemical reagent</th>
<th>Catalog number</th>
<th>Supplier</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine hydrochloride</td>
<td>G3272-500G</td>
<td>Sigma Aldrich</td>
<td>&gt;99.5%</td>
</tr>
<tr>
<td>HEPES</td>
<td>BDH4518-1KG9</td>
<td>VWR</td>
<td>&gt;99.9%</td>
</tr>
<tr>
<td>Imidazole</td>
<td>A10221</td>
<td>Alfa Aesar</td>
<td>99%</td>
</tr>
<tr>
<td>IPTG</td>
<td>1758-1400</td>
<td>Inalco Sas</td>
<td></td>
</tr>
<tr>
<td>Kanamycin sulfate</td>
<td>420311</td>
<td>Calbiochem</td>
<td></td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>BP214-500</td>
<td>Fisher Scientific</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>MES</td>
<td>BDH4544</td>
<td>VWR</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>Sigma Aldrich</td>
<td>99.8%</td>
</tr>
<tr>
<td>Monobasic sodium phosphate</td>
<td>BP330-1</td>
<td>Fisher Scientific</td>
<td>98%</td>
</tr>
<tr>
<td>Nickel (II) Sulfate</td>
<td>211085000</td>
<td>Acros Organic</td>
<td>99%</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>BDH0286-500G</td>
<td>VWR</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>15525-017</td>
<td>Gibco</td>
<td>&gt;99.5%</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>S612-3</td>
<td>Fisher Scientific</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>BP150-100</td>
<td>Fisher Bioreagents</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>BP982-100</td>
<td>Fisher Scientific</td>
<td></td>
</tr>
<tr>
<td>Tris base</td>
<td>BP152-5</td>
<td>Fisher Scientific</td>
<td></td>
</tr>
<tr>
<td>Tryptone</td>
<td>1211-1</td>
<td>Mo Bio Labs</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>BP169-500</td>
<td>Fisher Scientific</td>
<td>99%</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>BP565-10</td>
<td>Fisher Scientific</td>
<td>Biotech grade</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>DF0127071</td>
<td>Fisher Scientific</td>
<td></td>
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</table>

Table 2.2 Biological reagents, Enzymes and purification kits

<table>
<thead>
<tr>
<th>Enzymes and kits</th>
<th>Catalog number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>NS3231S</td>
<td>NEB</td>
</tr>
<tr>
<td>BglII</td>
<td>R0144S</td>
<td>NEB</td>
</tr>
<tr>
<td>Benzonase</td>
<td>70746</td>
<td>Novagen</td>
</tr>
<tr>
<td>Bio-Rad protein assay reagent</td>
<td>500-0006</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>BCA protein assay kit</td>
<td>23225</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>BL21(DE3) competent cells</td>
<td>69450</td>
<td>EMD Millipore</td>
</tr>
<tr>
<td>1 Kb DNA Ladder</td>
<td>N3232S</td>
<td>NEB</td>
</tr>
<tr>
<td>KOD Hot Start DNA Polymerase</td>
<td>71086-3</td>
<td>Novagen</td>
</tr>
<tr>
<td>MiniElute PCR Purification Kit</td>
<td>28004</td>
<td>Qiagen</td>
</tr>
<tr>
<td>NotI</td>
<td>R0189S</td>
<td>NEB</td>
</tr>
<tr>
<td>NcoI</td>
<td>R0193S</td>
<td>NEB</td>
</tr>
</tbody>
</table>
Table 2.2 Biological reagents, Enzymes and purification kits – Continued

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Catalog Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>NovaBlue competent cells</td>
<td>71227-3</td>
<td>Novagen</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>28706</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick Spin Miniprep Kit</td>
<td>27106</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Quick Change II Site-Directed Mutagenesis Kit</td>
<td>200521-5</td>
<td>Stragene</td>
</tr>
<tr>
<td>Rainbow Protein marker</td>
<td>RPN800E</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Rosetta (DE3) competent cells</td>
<td>70954-3</td>
<td>Novagen</td>
</tr>
<tr>
<td>Fast-Link DNA Ligation Kit</td>
<td>LK0750H</td>
<td>Epicentre</td>
</tr>
<tr>
<td>Gel Filtration Calibration Kit LMW</td>
<td>28-4038-41</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>TEV encoding plasmid</td>
<td></td>
<td>Kind gift from Dr. Timothy A. Cross, National High Magnetic Field Laboratory, Florida State University</td>
</tr>
<tr>
<td>ATP7B, CDNA for Wilson disease protein</td>
<td></td>
<td>Generous donation from Dr. Jonathan Gitlin, Marine Biological Laboratory, Wood Hole</td>
</tr>
</tbody>
</table>

Table 2.3 Proteins Notation (*Plasmodium falciparum* copper P-type ATPase)

<table>
<thead>
<tr>
<th>Notation</th>
<th>Protein name</th>
<th>Residue number</th>
<th>Number of base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfCuP-MBD1</td>
<td>N-terminal domain 1</td>
<td>1-70</td>
<td>210</td>
</tr>
<tr>
<td>PfCuP-MBD2</td>
<td>N-terminal domain 2</td>
<td>394-470</td>
<td>231</td>
</tr>
<tr>
<td>PfCuP-MBD2-3</td>
<td>N-terminal domain 2-3</td>
<td>394-564</td>
<td>513</td>
</tr>
<tr>
<td>PfCuP-MBD3</td>
<td>N-terminal domain 3</td>
<td>481-564</td>
<td>252</td>
</tr>
<tr>
<td>DdCuP-MBD1</td>
<td>N-terminal domain 1 with CX3C motif</td>
<td>205-272</td>
<td>204</td>
</tr>
<tr>
<td>DdCuP-MBD2</td>
<td>N-terminal domain 2 with CX2C motif</td>
<td>356-425</td>
<td>210</td>
</tr>
<tr>
<td>WLN1-3</td>
<td>N-terminal domains 1-3 of human ATP7B</td>
<td>57-327</td>
<td>813</td>
</tr>
<tr>
<td>WLN1-4</td>
<td>N-terminal domains 1-4 of human ATP7B</td>
<td>57-431</td>
<td>1125</td>
</tr>
<tr>
<td>WD4-HAH1</td>
<td>Chimera of WLN domain 4 and HAH1 chaperone</td>
<td>356-487 of ATP7B and 1-68 of HAH1</td>
<td>600</td>
</tr>
<tr>
<td>DdAtox1</td>
<td><em>Dictyostelium</em> discoideum copper metallochaperone</td>
<td>1-68</td>
<td>207</td>
</tr>
</tbody>
</table>
2.3 PCR primers

All PCR primers were purchased from Integrated DNA Technologies, Inc. Table 2.4 contains a list of all primers used for the gene amplification of various protein constructs.
Table 2.4 PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>bp</th>
<th>%GC</th>
<th>TM °C</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PfCuP</em>-D1 BamHI Fwd</td>
<td>54</td>
<td>51.9</td>
<td>70.3</td>
<td>CAGAGCGACGCCCATGGCAAAACTGTCGCTGACGATCAATAATGTGGACGATGAC</td>
</tr>
<tr>
<td><em>PfCuP</em>-D1 BamHI Rev</td>
<td>54</td>
<td>50</td>
<td>70.1</td>
<td>CGCAAGCTTGGCGGATCCATGCTATTTGCTGCTAATGTGACGCGGTCGCTTCAG</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 Fwd N-terminal His tag</td>
<td>60</td>
<td>58.3</td>
<td>74.4</td>
<td>CGACGCATGCGCCATGGATGCATCAACCACACCACACCACAGCGGGATTGAGGTCGCGAAAAT</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 Reverse primer</td>
<td>43</td>
<td>53.5</td>
<td>68.2</td>
<td>CGCGGATCCGCCTCACTATGTGCAACTCGAAGGTCAAGTACGAGATCAATCTAC</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 Forward primer</td>
<td>42</td>
<td>47.6</td>
<td>66.3</td>
<td>CGGACTGCGCATTTTACATCTCGAAGTCAGTACACTAC</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 Reverse primer</td>
<td>60</td>
<td>43.3</td>
<td>68.3</td>
<td>CGCGGATCCCTCAGTGGTGATGTTGCGGATGACCTTGAAAATAAGATTTTCGTCGCTTCATTTT</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 With Trx fusion tag Forward primer</td>
<td>60</td>
<td>38.3</td>
<td>65.9</td>
<td>GGTATTGAGGGTTCGCGAAAAATCTATATTTTCAAGGTTACATCTCGAAGTAAATCTAC</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 Reverse primer</td>
<td>41</td>
<td>46.3</td>
<td>64.3</td>
<td>AGAGGAGAGTTCGATGCGCCTCAGTCATCTTTTACAGGTTACGAGGTCGCTAC</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 and PfCuP-D2-3 Poly glycine Forward primer</td>
<td>45</td>
<td>57.8</td>
<td>71.3</td>
<td>CTATATTTTCAAAGGGTGGCAGCGGAGCGGGCTACATCTCGGAACTG</td>
</tr>
</tbody>
</table>
| *PfCuP*-D2 and PfCuP-D2-3 Poly glycine Reverse primer | 45 | 57.8 | 71.3 | CAGTTCGCAGATGTTAGCCGGCGCCGCGCCGCCACCTTGAAAATAAGT
Table 2.4 PCR primers – Continued

<table>
<thead>
<tr>
<th>Primer Description</th>
<th>Tm (°C)</th>
<th>GC (%)</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PfCuP</em>-D2 and <em>PfCuP</em>-D2-3 Insert three glycine</td>
<td>36</td>
<td>63.9</td>
<td>CTATATTTCAGGTCGCGGCTACATCTGCGAACTG</td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 and <em>PfCuP</em>-D2-3 Insert three glycine</td>
<td>35</td>
<td>63.9</td>
<td>CAGTTTCAGATGTAGCCGACCTTTGAAAATATAG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 and <em>PfCuP</em>-D2-3 Insert Threonine at P2</td>
<td>33</td>
<td>60</td>
<td>CTATATTTCAGGTCGCTACATCTGCGAACTG</td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 and <em>PfCuP</em>-D2-3 Insert Threonine at P2</td>
<td>33</td>
<td>60</td>
<td>CAGTTCCAGATGGTACCTTGGAACCTG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 and <em>PfCuP</em>-D2-3 Insert Valine at P2</td>
<td>33</td>
<td>58.6</td>
<td>CTATATTTCAGGTCGCTACATCTGCGAACTG</td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2 and <em>PfCuP</em>-D2-3 Insert Valine at P2</td>
<td>33</td>
<td>58.6</td>
<td>CAGTTTCAGATGTAGACACCTTGGAATTAGAATATAG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2-3 C-terminal His tag</td>
<td>42</td>
<td>66.3</td>
<td>CGGACGCATGGCATGGATTACATCTGCGAACTGAAATCTAC</td>
</tr>
<tr>
<td>Forward primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PfCuP</em>-D2-3 C-terminal His tag Reverse primer</td>
<td>60</td>
<td>68.1</td>
<td>CGCGGATCTCAGTGGATGGTTGGATGGTGACCTTTGAAAATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TAGATTCCGTCTGTGTTTGT</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2-3 With TRX fusion tag, Forward primer</td>
<td>60</td>
<td>65.9</td>
<td>GGTATTGAGGAGTCCGCGAAAATCTATATATATTTCAAGGTTACATC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TGCGAACTGGAATATCTAC</td>
</tr>
<tr>
<td><em>PfCuP</em>-D2-3 With TRX fusion tag, Reverse primer</td>
<td>41</td>
<td>66.5</td>
<td>AGAGGAGAGTATTAGACCTCATCTGCTTTTGTGAGGAGCG</td>
</tr>
<tr>
<td>Primer Set</td>
<td>Forward/Reverse</td>
<td>Tm</td>
<td>Seq (5’ → 3’)</td>
</tr>
<tr>
<td>------------</td>
<td>----------------</td>
<td>----</td>
<td>---------------</td>
</tr>
<tr>
<td><em>PcfCuP-MBD3</em> Forward primer</td>
<td>54</td>
<td>50</td>
<td>69.9</td>
</tr>
<tr>
<td><em>PcfCuP-MBD3</em> Reverse primer</td>
<td>48</td>
<td>50</td>
<td>68.9</td>
</tr>
<tr>
<td>Wilson protein Forward primer:</td>
<td>55</td>
<td></td>
<td>71.6</td>
</tr>
<tr>
<td>Wilson protein Reverse primer (with Not1):</td>
<td>60</td>
<td>53.3</td>
<td>72.1</td>
</tr>
<tr>
<td>Wilson protein Reverse primer (with BamH1):</td>
<td>60</td>
<td>46.7</td>
<td>68.6</td>
</tr>
<tr>
<td>HAH1 Xma1 mutation Forward</td>
<td>39</td>
<td>53.8</td>
<td>68.3</td>
</tr>
<tr>
<td>HAH1 Xma1 mutation Reverse</td>
<td>39</td>
<td>53.8</td>
<td>69.3</td>
</tr>
<tr>
<td>WD4 Xma1 mutation Forward</td>
<td>33</td>
<td>57.6</td>
<td>69.3</td>
</tr>
<tr>
<td>WD4 Xma1 mutation Rverse</td>
<td>33</td>
<td>57.6</td>
<td>69.3</td>
</tr>
<tr>
<td>HAH1 Forward</td>
<td>62</td>
<td>61.3</td>
<td>74.7</td>
</tr>
<tr>
<td>HAH1 Reverse</td>
<td>43</td>
<td>62.8</td>
<td>73.3</td>
</tr>
</tbody>
</table>

TRX: Thioredoxin fusion tag
P2: Position two of TEV protease recognition site (ENLYFQG.), Glycine residue (G) is at position 1
Poly Glycine: Six Glycine residues
2.4 Instrumentation

This section describes the instruments their usage and the source of the instruments.

These details are described in tables 2.5 and 2.6

Table 2.5 Instrumentation, source and usage

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Usage</th>
<th>Model</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKTA FPLC</td>
<td>Protein purification</td>
<td>18-1900-26</td>
<td>GE life sciences</td>
</tr>
<tr>
<td>AKTA Prime plus</td>
<td>Protein purification</td>
<td>03-0014-96</td>
<td>GE life sciences</td>
</tr>
<tr>
<td>Analytical balance</td>
<td>Weight measurements &gt; 0.1mg</td>
<td>AT400</td>
<td>Mettler Toledo</td>
</tr>
<tr>
<td>Analytical balance</td>
<td>Weight measurements &gt; 0.1g</td>
<td>PG603-SDR</td>
<td>Mettler Toledo</td>
</tr>
<tr>
<td>CD Spectrophotometer</td>
<td>Circular Dichroism measurements</td>
<td>J-815</td>
<td>JASCO</td>
</tr>
<tr>
<td>Glove box</td>
<td>Performing experiments under anaerobic conditions</td>
<td></td>
<td>Vacuum atmosphere NEXXUS chamber under nitrogen</td>
</tr>
<tr>
<td>MiniCycler</td>
<td>PCR</td>
<td>MJ research</td>
<td>PTC 150</td>
</tr>
<tr>
<td>NMR facility at Michigan State University</td>
<td>'H-'N HSQC and Triple resonance experiments</td>
<td>Varian</td>
<td>Unity Inova 600 MHZ</td>
</tr>
<tr>
<td>NMR facility at the University of Notre Dame</td>
<td>'H-'N HSQC experiments</td>
<td>Bruker</td>
<td>AVANCE II, 800 MHZ</td>
</tr>
<tr>
<td>Refractometer</td>
<td>Determination of [GuHCl]</td>
<td>Bausch and Lomb</td>
<td>Abbe 3L</td>
</tr>
<tr>
<td>Freezer</td>
<td>Storage of samples at -80°C</td>
<td>HARRIS</td>
<td>B65066A</td>
</tr>
<tr>
<td>pH benchtop meter</td>
<td>pH measurements</td>
<td>Thermo electron corporation</td>
<td>Orion 3 star pH benchtop</td>
</tr>
<tr>
<td>Shaker/Incubator</td>
<td>Growth of bacterial culture</td>
<td>New Brunswick Scientific</td>
<td>Innova 4300</td>
</tr>
<tr>
<td>Gravity Convection Incubator</td>
<td>Growth of bacterial colonies</td>
<td>ICB 0052</td>
<td>Precision</td>
</tr>
<tr>
<td>UV-VIS Spectrophotometer</td>
<td>Quantification of DNA and Protein in solution</td>
<td>DU-7400</td>
<td>Beckman</td>
</tr>
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</table>
### Table 2.6 Columns used for protein purification (GE Life Sciences)

<table>
<thead>
<tr>
<th>Column</th>
<th>Usage</th>
<th>Resin</th>
<th>Part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM Sepharose XK 26</td>
<td>Cation exchange purification</td>
<td>Carboxy methyl sepharose</td>
<td>56-1053-34</td>
</tr>
<tr>
<td>DEA Sepharose XK 26</td>
<td>Anion exchange purification</td>
<td>Diethyl amino sepharose</td>
<td>56-1053-34</td>
</tr>
<tr>
<td>HiPrep 26/10 Desalting column</td>
<td>Buffer exchange</td>
<td>Superdex G-25 superfine</td>
<td>17-5087-01</td>
</tr>
<tr>
<td>HisPrep FF 16/10</td>
<td>His tag affinity purification</td>
<td>Ni Sepharose 6 Fast Flow</td>
<td>28-9365-51</td>
</tr>
<tr>
<td>HiLoad 26/60 Superdex 75 pg</td>
<td>Size exclusion chromatography</td>
<td>Superdex 75 Prep grade</td>
<td>17-1070-01</td>
</tr>
<tr>
<td>HiLoad 16/600 Superdex 75 pg</td>
<td>Determination of apparent molecular weight</td>
<td>Superdex 75 Prep grade</td>
<td>28-9893-33</td>
</tr>
</tbody>
</table>

### Table 2.7 Plasmids used for the protein constructs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pET24d(+)</td>
<td>Expression of protein without fusion tag</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET28b(+)</td>
<td>Expression of protein with C-terminal His tag</td>
<td>Novagen</td>
</tr>
<tr>
<td>α-pET28a (+)-α</td>
<td>Expression of protein with a N-terminal YaiN (α) fusion tag</td>
<td>Kind gift from Prof. Nathan Nelson Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel</td>
</tr>
<tr>
<td>pET28a (+)-α</td>
<td>Expression of protein with a C-terminal YaiN (α) fusion tag</td>
<td></td>
</tr>
<tr>
<td>α-pET28a (+)-α</td>
<td>Expression of protein with a both N and C-terminal YaiN (α) fusion tags</td>
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</tr>
<tr>
<td>α-pET28a (+)-β</td>
<td>Expression of protein with a N-terminal YaiN (α) and C-terminal YbeL (β) fusion tags</td>
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<tr>
<td>β-pET28a (+)-α</td>
<td>Expression of protein with a N-terminal YbeL (β) and YaiN (α) fusion tags</td>
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<tr>
<td>β-pET28a (+)-β</td>
<td>Expression of protein with a N-terminal YbeL (β) and C-terminal YbeL (β) fusion tags</td>
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Table 2.7 – Continued

<table>
<thead>
<tr>
<th>Expression of protein with a N-terminal YbeL (β) fusion tag</th>
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<td>Expression of protein with a C-terminal YbeL (β fusion tag</td>
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<tr>
<td>Expression of Thioredoxin fusion tagged proteins</td>
<td>pET32Xa/LIC</td>
</tr>
</tbody>
</table>

2.5 Amino acid sequences

2.5.1 *Plasmodium falciparum* PfCuP-ATPase constructs

PfCuP-D1 Amino Acid Sequence

```
1MAKLSLTINNVDDDLKRKIKKAKFEGVEDLKHKNKLTISYNPKVVDSYGVIERIKHLDINGEILKDDNDISSK
```

Length; 70 residues, MW; 8066 Daltons

Figure 2.1 Amino acid sequence of *PfCuP*-D1

PfCuP-MBD2 Amino Acid Sequence

```
394YICELKIYNMTCDNGKKIINFLKDKNLILEGNSFATDNKIKKLIDSSDHICN
478NVMFVNTIMNEIKESGFNdNDLddLYKDD
```

Length; 84 residues, MW; 9854 Daltons

Figure 2.2 Amino acid sequence of *PfCuP*-MBD2

PfCuP-D3 Amino Acid Sequence

```
477DDKNRCNLSEITLYIYRDDIKKSYKLLKDKGISNVEYDLKKEIYFLYDPELVGI
564RYILEILKKKNVDAyyYDEdKEKFRSSQNNE
```

Length; 88 residues, MW; 10779 Daltons

Figure 2.3 Amino acid sequence of *PfCuP*-D3
Figure 2.4 Amino acid sequence of PfCuP-D2-3. PfCuP-D2 (green), linker (blue), PfCuP-D3 (red), linker between PfCuP-D3 and the first predicted transmembrane domain (purple).

2.5.2 Dictyostelium discoideum ATP1 constructs

```
YICELKIYNTCDNCGKKIINFKLKDDKKLLEGNSFATDNKIKLKIIDISSDIHCNNV
KMVFNTIMEKESGFNNDDLLYKDDKNCNLSEITLILYRDDIKKSYKOLLKDIDG
SNVEYDLKKEYIYFLYDPELVGIRYILEILKKKKNVDAYYDDEKEKYFRSSQNN
```

Length; 171 residues, MW; 20385 Daltons

Figure 2.5 Metal binding domain 1 of Dictyostelium discoideum ATP1 amino acid sequence

```
SRERVFEIKGMKEVGCANRIEATLLQNKIHSVKINFKTQQLFVIAVNDRTIRKTI
EKLGFKCQKQ
```

Length; 69 residues, MW; 7938 Daltons

Figure 2.6 Metal binding domain 2 of ATP1 amino acid sequence

```
IISIGVFGMTASCVGMVEHSIKSVDGVIENCVNLLAERAIYKNSSICKDVKEIQESI
EILGFETKLIQSSKAG
```

Length; 75 residues, MW; 8176 Daltons

2.5.3 Dictyostelium discoideum Atox1

```
MGTYSFVDMTCGGCSKAHVAINLSKIDGVSNIQDLENNKVSCESSKMADELLKNIQ
KTGKKCSIPIA
```

Length; 69 residues, MW; 7263 Daltons

Figure 2.7 DdAtox1 amino acid sequence

2.5.4 Chimera of Wilson protein domain 4 and HAH1(WD4-HAH1)

```
GTCSTTLIAIAGMTASCVHSIEGMSQUELVQQISVSLAEGTAVLYNPASPEELRA
AIEDMGEASVVESTNLPNSAGAMVQTTDDGTPTSLQEVAPHTGRLPAH
DILAKSPQSTRAVMPKESVDTCGGCAEAVSRVNLKLGVKYIDLPNKKVCIES
EHSMDTLATKKTGKTVSYLGE
```

Length; 199 residues, MW; 20677 Daltons

Figure 2.8 WD4-HAH1 amino acid sequence
2.6 Procedures

2.6.1 Plasmids construction, protein expression and purification

2.6.1.1 Plasmid construction of a full length ATP7B

Overexpression of membrane proteins (MP) in *E. coli* is challenging. In fact, the expression of membrane protein in *E. coli* often leads to their aggregation and reduced levels of host membrane and secretory proteins.\(^1\) MP toxicity is due to the overloading of the Sec-dependent translocation machinery, which handles both the post-translational export of secretory proteins and the co-translational insertion of most inner MP into the lipid bilayer.\(^2\) One approach, however, has been to make use of *E. coli* strains C41 (DE3) and C43 (DE3), which tolerate the accumulation of toxic proteins. Another common method that can enhance overexpression of MP in *E. coli* is the use of fusion proteins.\(^3,4\)

The genetic fusion of the protein of interest to the N or C termini of other proteins can yield higher expression, increase solubility and simplify detection and affinity purification of the overexpressed products.\(^3\)

Full length ATP7B has 1465 amino acid residues. The gene containing the ATP7B was amplified by PCR using primers designed such that the first 57 residues were omitted. These residues were removed since they were predicted to be unstructured. The gene was amplified from pCDNA containing the full length ATP7B gene. The PCR reaction was done using the KOD Hot Start DNA polymerase protocol, Table 2.63 (a) and (b). The primers were designed such that either the forward, or reverse primers encodes for the Tobacco Etch Virus (TEV) protease recognition site. Both forward and reverse primers include a sequence compatible with pET28a (+) vector. pET28a (+) derived expression vectors were originally engineered by Leviatan *et al.*\(^5\) to contain bacterial fusion domains.
at either the N, C or both termini of the gene of interest. This expression cassette for proteins was expected to increase the chances that membrane protein overexpression will be successful. The fusion domains are two short hydrophilic bacterial proteins \textit{YaiN} and \textit{YbeL}, (denoted as \(\alpha\) and \(\beta\)) proteins, respectively. When fused to the end of membrane proteins they serve as facilitators for expression and purification. The \(\alpha\) and \(\beta\) domains are hydrophilic and possess globular structure. The \(\alpha\) fusion domain belongs to the \textit{E.coli} operon \textit{frm}, which is likely involved in the degradation of formaldehyde.\(^6\) The \(\beta\) fusion domain is composed of the first 120 amino acid residues of the \textit{E. coli} \(\beta\) gene that has no known function.\(^7\) The PCR product for the ATP7B gene that was inserted into pET28a(+) was 4235 bp, and was purified by gel extraction kit from Qiagen to remove the background products.

2.6.1.2 Cloning of the ATP7B gene into pET-28 expression plasmids

The cloning of the ATP7B gene was designed such that the gene is inserted into the respective pET28a(+) derived vectors with the NotI and BamHI recognition site. The scheme for the ligation into these vectors is illustrated in the Figure 2.64

![Diagram](image)

Figure 2.9 Organization used to clone the WDP gene into pET 28a (+) vectors.
vector plasmids and the PCR products were digested with BamHI and NotI restriction enzymes. The digestion reaction was incubated at 37°C for three hours and the vector DNA purified by gel extraction using Qiagen gel extraction kit protocol and the digested PCR insert DNA was product purified using Qiagen PCR purification kit. The purified DNA samples were assessed by agarose gel electrophoresis to gauge purity and success of the digestion (Figure 2.65).

![Figure 2.10](image)

Figure 2.10 (a) M; 1 kb DNA marker, 1 &2; PCR product for ATP7B. (b) M; 1kb DNA marker; 1 &2 purified and digested PCR product, 3; purified and digested vector DNA.

### 2.6.1.3 Ligation reaction

The DNA insert was treated with T4 DNA ligase and incubated at room temperature for 20 min. The ligase was deactivated by incubating the reaction mixture at 70 °C for 10 min. The vector: insert ratio used for the ligation was 1:3. If 120 ng of vector DNA was used, the amount of the insert DNA needed was calculated as follows:

\[
\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb of vector}} \times \frac{\text{insert}}{\text{vector}} = \text{ng of insert}
\]

\[
\frac{120 \text{ ng} \times 4.235 \text{ kb}}{5.7 \text{ kb}} \times \frac{3}{1} = 267.47 \text{ ng of insert}
\]
2μL of the ligation reaction was then used to transform GigaSingles NovaBlue competent cells and incubated overnight at 37°C. A single colony was inoculated into 5 mL LB medium containing 50 μg/ml of kanamycin and grown at 37°C for 16 hours. The plasmids were purified using Qiagen plasmid purification kit protocol. Restriction mapping and agarose gel electrophoresis confirmed plasmids with inserts in the correct orientation (Figure 2.67 C and D). DNA sequencing by Retrogen, Inc. (San Diego, CA) further confirmed successful clones.

2.6.1.4 Induction test for the ATP7B protein expression

Plasmids containing full length ATP7B (minus the first 57 amino acids), were used to transform Rosetta2(DE3) competent cells. Single colonies from an overnight growth were used to inoculate 5 mL LB medium with kanamycin (5 μg/mL final concentration). The culture was grown at 37°C with shaking and induced with IPTG (1mM final concentration) at O.D₆₀₀ = 0.6 – 0.8. Protein expression was carried out for four hours at 37°C. Samples were taken hourly for four hours and protein expression assessed by SDS gel electrophoresis. The expression of the full length ATP7B protein in Rosetta2(DE3) cells was not evident in the absence of antibody detection. Lower molecular weight bands appeared, but their origin needed to be verified by MALDI-TOF or Q-TOF mass spectrometry (~ 40 kDa, Figure 2.67 C lanes 4-7).

Protein expression at lower temperatures, 16°C - 25°C was also attempted, but the temperature reduction did not improve the yield. Different E. coli strains such as BL21(DE3), C41(DE3), C41 pLysS, C43 and C43 pLysS were used for protein expression. It was observed that when expression of the ATP7B protein was induced,
most of the BL21(DE3) cells died. This was seen from a reduction in O.D.\textsubscript{600} C41(DE3) and C43(DE3), which are derivatives of BL21(DE3), are known to be effective in expression of membrane proteins and toxic proteins\textsuperscript{8,9} and were thus used for the induction. Induction test with C41 and C43 cells at 18\textdegree C for 16 hours yielded a higher molecular weight band of (~ 150 kDa, Figure 2.67 D lanes 3, 4 and 7, 8, 9 respectively).

2.7 Summary

Wilson disease protein has been cloned into variants of pET28a(+) plasmids for the expression in \textit{E. coli}. Successful cloning was confirmed by DNA sequencing. Protein expression at different temperature was attempted with different \textit{E. coli} cell strains. C41 and C43 were able to express the protein at small scale and at 18\textdegree C. However, attempts to scale up the expression were not successful. Future experiment may be designed in such a way that either the ATP7B gene be synthesized to take advantage of codon
optimization for the expression in \textit{E coli}, alternatively different expression systems could be used such as expression of the protein in mammalian cells or yeast.
2.8 REFERENCES


7. Perna, N. T.; Plunkett, G., 3rd; Burland, V.; Mau, B.; Glasner, J. D.; Rose, D. J.; Mayhew, G. F.; Evans, P. S.; Gregor, J.; Kirkpatrick, H. A.; Posfai, G.; Hackett, J.; Klink, S.; Boutin, A.; Shao, Y.; Miller, L.; Grotbeck, E. J.; Davis, N. W.; Lim,


CHAPTER 3

BIOPHYSICAL PROPERTIES OF WD4-HAH1 CHIMERA

3.1 Introduction

Previous attempts to trap, and characterize the copper-bridged intermediate between WD4 and HAH1(Atox1) have not succeeded. Entropically linking these two protein together may increase the residence time of the interaction or increase the number of bridged intermediates within a given time frame. This can be assessed by a number of techniques such as XANES/EXAFS, NMR, X-ray crystallography, gel filtration and light scattering. The construction of the chimera utilized the native amino acids sequence that follows after WD4, thus attempting not to perturb the native structure of the protein.

3.2 Construction of WD4-HAH1 expression plasmid

WD4-HAH1 is a chimera of two proteins (WD4 of ATP7B and HAH1, a copper chaperone for ATP7B). The chimera was created by cloning HAH1 at the C-terminal end of WD4 such that the two proteins are separated by a linker. This was achieved by creating a XmaI site at the C-terminus of WD4 by mutating two amino acid residues (A432P and P433G) of domain 5 of ATP7B. A plasmid in which domains four through six had been previously cloned by Dr. Ibtesam Alja’afreh into a pET-32 Xa/LIC vector, here referred to as pET-32 Xa/LICWD4-6, was used. The HAH1 gene had previously been cloned into pET-11d vector by Dr. David Huffman at Northwestern University.\(^1\) This plasmid was used as a template for PCR amplification and HAH1 mutations. A mutation was made to remove the XmaI site that is present in HAH1 gene and subsequently create a new XmaI site by

mutagenesis when the gene was amplified by PCR. The PCR product was purified using the QIAGEN PCR purification kit and the DNA was prepared for cloning by digestion with the XmaI restriction enzyme. Both the purified vector (pET-32 Xa/LIC WD4-6) and HAH1 PCR product were digested with the XmaI restriction enzyme by incubating at 37 °C for 4 hrs. The vector DNA was dephosphorylated using Shrimp Alkaline Phosphatase and purified by gel extraction (QIAGEN). The digested HAH1 DNA was purified using QIAGEN PCR purification kit and its purity assessed using agarose gel electrophoresis (Figure 2.69).

![Figure 3.1 PCR product for HAH1 (210 bp) L; 1kb DNA marker, 1&2 HAH1 PCR product](image)

HAH1 and the vector DNA containing WD4 were cloned using a molar ratio of 3:1 (insert: vector) with T4 DNA ligase. The ligation reaction was performed at room temperature for 1 hour One tenth of the ligation reaction was used to transform NovaBlue competent cells. Colonies were grown overnight at 37°C on LB plates with ampicillin (100 μg/mL final concentration). Colonies were then screened for successful clones and the plasmid (pET-32- WD4-HAH1) with the correct insert were confirmed by DNA sequencing. Figure 2.68 shows a flow diagram of cloning HAH1 into WD4.
Figure 3.2 Flow diagram of WD4-HAH1 construct
3.3 Induction test for WD4-HAH1 protein expression

PET32-WD4-HAH1 plasmid was transformed into Rosetta2(DE3) competent cells for small scale protein expression. Five colonies were screened for protein expression. Each colony was inoculated in 5 mL LB with Ampicillin and incubated at 37°C (until O.D$_{600}$ = 0.6-0.8). Cells were then induced with 1 mM IPTG and incubated for an additional 4 hours with shaking (250 rpm). Some samples were induced at 30°C for 4-6 hrs. 300 µL of culture was centrifuged to obtain pellet. The pellets were then prepared for SDS-PAGE (Figure 3.3).

![Figure 3.3 Small scale protein expression at 30°C for 4-6 hours. Lane M: protein marker, Lane 1: 0 hours induction, Lane 2: 1 hour induction, Lane 3: 2 hours induction, Lane 4: 3 hours induction, Lane 5: 5 hours induction.]

3.4 Protein expression and purification of WD4-HAH1

Rosetta2(DE3) competent cells were transformed with pET-32-WD4-HAH1 plasmid. Colonies were grown to density at 37°C in 5 mL LB/ampicillin. This starter culture was then scaled up to 1 liter LB/ampicillin and incubated in a shaker at 37°C until the O.D$_{600}$ = 0.6-0.8. Cells were then induced with 1mM IPTG and incubated for four hours with shaking at 250 rpm. After four hours the cells were harvested by centrifugation at 4°C for
30 minutes at 15,000 rpm. The pellet was stored at -20°C. The pelleted bacteria were freeze-thawed in liquid nitrogen 5 times and re-suspended in phosphate lysis buffer at pH 7.4 (20 mM NaH₂PO₄, 100 mM NaCl, 2 mM MgCl₂, 20 mM imidazole, 0.5 mM DTT). 1µL Benzonase was added and re-suspended cells were mixed by gently shaking at 4°C for 30 minutes. The clarified supernatant was obtained centrifuging at 15000 rpm for 30 minutes to obtain the supernatant.

3.5 His Trap purification of Trx-WD4-HAH1

The supernatant was filtered and loaded onto a HisTrap column (GE life science, 20 mL). Prior to loading the sample, the column was washed with seven column volumes of elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 7.5). The column was equilibrated with seven column volumes of binding buffer (20 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, pH 7.5) prior to loading the sample. The bound protein was eluted by gradient elution with five column volumes of elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 7.5).

3.6 Gel filtration of Trx-WD4-HAH1

The protein was further purified using a HiLoad 26/60 Superdex 75 gel filtration column. Fractions of HisTrap elution were combined, concentrated to 9 mL and loaded onto a gel filtration column that had been pre-equilibrated with 2 column volumes of phosphate buffer
(50 mM NaH₂PO₄, 200 mM NaCl, 10 mM DTT, pH 7.5). The protein sample was eluted with 1.2 column volumes of the same buffer and purity of the protein was assessed using SDS-PAGE (Figure 3.6). After gel filtration the protein was desalted with Tris-Cl (50 mM Tris, 0.5 mM EDTA, 1 mM DTT, pH 8.0).

![Figure 3.4. SDS-PAGE for WD4-HAH1](image)

Lane 1; pellet, lane 2; extract, lane 3-8; His Trap elution fractions, lane 9; gel filtration fraction, lane L; marker.

### 3.7 TRX-WD4-HAH1 digestion with TEV protease

WD4-HAH1 was expressed with a TEV-removable thioredoxin fusion tag. A TEV protease cleavage site was inserted at the N-terminus of WD4 during cloning, and enables the removal of the thioredoxin tag. The protein sample was then mixed with TEV at a mass ratio of 80:1 (WD4-HAH1: TEV protease) and incubated for 12 hours at room temperature. The TEV digested protein was then purified with HisTrap column to remove the fusion tag. The digestion and the purity of the protein was assessed using SDS-PAGE (Figure 3.7).
3.8 Biophysical characterization of WD4-HAH

3.8.1 Introduction

Wilson disease protein (ATP7B) is a trans-membrane protein involved in cellular copper metabolism. To date, the complete structure of the protein has not been solved. However, structures of the individual domains have been elucidated. The N-terminal portion, composed of 633 amino acid residues, has six soluble metal binding domains, that have a ferredoxin-like fold and plays an important role in the copper acquisition. Each domain also has a conserved GMT/HCXXC copper binding motif, and the two cysteine residues coordinate the metal ion. The respective metal binding domains are separated by linkers. Domains 1 and 2 are separated by 12 residues; domains 2 and 3 are separated by a 40 residue linker; domains 3 and 4 have a 30 residue while domain 4 and 5 are separated by a 56 residue linker; domain 5 and 6 have the shortest linker of 8 amino acids residues (Figure 3.5 Purification of WD4-HAH1. 1; Undigested Trx-WD4 Hah1 (with thioredoxin fusion tag), 2 &3; Trx-WD4-HAH1 +TEV incubated for 6 hours and 12 hours respectively, 4 WD4-HAH1 His trap fraction after TEV digestion, 5; pure WD4-HAH1 after gel filtration, M; protein marker.)
1.27). The structures of the metal binding domain of the ATP7B and the Menkes disease protein have been solved by NMR and X-ray.\textsuperscript{2,3,4,5}

![Image](image.png)

Figure 3.6 Solution structure of WLN3-4 (PDB: 2ROP).

The interaction of the copper chaperone HAH1 with the metal binding domains has been studied by solution NMR.\textsuperscript{6,7,8} The six domains can receive copper from HAH1. Domain 6 can receive copper(I) from the chaperone without formation of an adduct. Domain 2 and 4 can simultaneously be loaded with copper(I) by the HAH1 chaperone with the formation of copper dependent adducts.\textsuperscript{8} Isolated domain 5-6 can receive copper(I) from domain 4 but not from HAH1.\textsuperscript{3} This implies not all domains receive copper from or form adducts with HAH1, but may receive the metal ion from other domains. Other species, such as yeast and bacteria, have fewer domains.\textsuperscript{9} Banci et al. showed that a construct of WLN3-4
was able to form a complex with Cu(I)HAH1. In the WLN3-4 construct, only domain 4 could form a stable adduct with Cu(I)HAH1 even though copper is efficiently transferred to both the domains. A similar observation was made with the Menkes protein domain in which both domain 3 and 4 were metallated by HAH1 with only domain 4 forming a stable adduct. Domain 3 can be metallated by either Cu(I)HAH1 through weak interactions or from metallated domain 4. The fact that not all domains form stable adducts with the HAH1, implies that the domains have different surface characteristics, including their interaction with the copper donor (HAH1 or other domains within the N-terminal portion of the protein). It has been previously shown that domain four of ATP7B is the most stable domain and that HAH1 may preferentially donate the copper(I) ion to this domain instead of the other five domains. Even though HAH1 is known to form a stable adduct with domain 4, the interactions between these two proteins has not been fully elucidated.

3.8.2 WD4-HA1 chimera

The chaperone HAH1 has the same βαββαβ protein fold as the MBDs in ATP7B. The transfer of Cu(I) to the MBD is dependent on the weak protein interactions between the CXXC metal binding motifs of the two proteins. The MBDs location within the N-terminal tail, electrostatic potentials and ability to orient in the correct direction may determine complex formation with the chaperone HAH1. HAH1-MBD and MBD-MBD interactions are very important in the function of the ATP7B; however, characterization of the transient interactions has been challenging. Several methods have been used to investigate the interaction of the chaperone HAH1 with the respective domains in ATP7A and ATP7B. These include surface plasmon resonance, NMR, X-ray crystallography,
molecular dynamic (MD) simulations\textsuperscript{13,14} protein docking\textsuperscript{15} and single molecule fluorescence resonance energy transfer (smFRET).\textsuperscript{16,17,18} Benitez et al. used smFRET method to study the interaction of two proteins by encapsulating the chaperone HAH1 and MBD4 of ATP7B in a nanovesicle both in the presence and absence of Cu(I). Their study revealed that HAH1 interacts with MBD4 in two different geometries which can interconvert dynamically.\textsuperscript{17} The multiple geometries may exist in order to increase the probability of complex formation that leads to the copper transfer from the chaperone to the metal binding domain.\textsuperscript{17,19}

Even though a number of studies have described the role of interactions in copper transfer from the chaperone to the individual metal binding domains of ATP7B, no structure has been reported for the complex formed between MBD4 and HAH1. In order to study the complex of metal binding domain four of ATP7B and the HAH1 chaperone, the two proteins were joined together to form a chimera referred to as WLN4-HAH1. The two proteins are separated by a 55 residue linker that joins domain 4 and 5 in the native ATP7B sequence.

\textbf{3.8.3 WD4-HAH1 Mass spectrophotometry}

The molecular weight of the WD4-HAH1 protein is 20.677 kDa as predicted using ExPasy based on its protein sequence (Figure 3.7). SDS-PAGE results were consistent with this mass. The exact molecular weight was verified by mass spectrophotometry,
which revealed the molecular weight to be 20,687kD (Figure 3.83).

![Figure 3.7 ESI-MS spectra for WD4-HAH1.](image)

3.8.4 Circular dichroism of WD4-HAH1

The purified protein samples were buffer exchanged into 30 mM sodium phosphate, 150 mM NaCl, pH 7.5. Protein concentration was determined using the BCA assay. A 20 μM protein concentration was used for CD measurements. The wavelength scan showed that
the α helix content of the protein is 28% (Figure 3.84). All measurements were done on a JASCO J-815 CD Spectropolarimeter.

3.8.5 Chemical unfolding of WD4-HAH1

Guanidine hydrochloride (GuHCl) was used for the chemical denaturation of WD4-HAH1. The concentration of GuHCl was determined using the refractive index for accurate concentration determination according to equation 3.

\[
C = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.6(\Delta N)^3
\]

Eq. 3

Where \( C \) is the concentration of the GuHCl in moles/L and \( (\Delta N) = \) (refractive index of solution minus refractive index of water). Protein samples were mixed with varying concentrations of GuHCl (0 – 7.5 M) and incubated at room temperature for two hours. The CD unfolding signal was followed at 222 nm. The CD data was fit in a three state unfolding model using Origin®. The chimera protein unfolds with more than one transition (Figure 3.85). The double sigmoidal curve corresponds to the unfolding of the HAH1 in
the first transition followed by the unfolding of WD4 in the second transition. Table 3.8 summarizes the free energies of unfolding of WD4-HAH1 chimera.

Table 3.8. Free energy of unfolding of WD4-HAH1

<table>
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<th></th>
<th>1st transition</th>
<th>2nd transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔG°_{H_2O} (kJ/mol)</td>
<td>m_i (kJ/mol)</td>
<td>c_m (M)</td>
</tr>
<tr>
<td>6.11 ± 0.56</td>
<td>1.05 ± 0.3</td>
<td>1.37 ± 0.02</td>
</tr>
<tr>
<td>44.8 ± 2.4</td>
<td>9.8 ± 1.2</td>
<td>5.61 ± 0.03</td>
</tr>
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The free energy of unfolding for the first transition was found to be 6.1 ± 0.56 kJ/mol while that of the second transition was 44.8 ± 2.4 kJ/mol. The GuHCl mid-point where F_D = F_N for the first transition was determined to be 1.37 ± 0.02 M, and that of the second transition was 5.61 ± 0.03 M.
3.8.6 Thermal unfolding of WD4- HAH1

Thermal unfolding was followed at 222 nm and the temperature gradually increased from 25°C to 98 °C. It was determined that the chimera is very thermally stable and has a high T_m about 78.1 ±0.3°C (Figure 3.86).

3.8.7 Crystallization of WD4-HAH1

Protein crystallization requires a supersaturated sample of protein. Crystals were obtained using the hanging drop vapor-diffusion method with 1:1(v: v) protein (10 mg/mL) and reservoir solution mixture at room temperature. In this method, a drop of a mixture of protein and precipitant is sealed in a chamber with the precipitant. As the water evaporates from the drop, vapor equilibrium is achieved between the protein and the reservoir solution causing protein crystals to begin to form in a crystal nucleation zone. Initial screening was performed using the Hampton 96 well HR2-110 and HR2-112 screening kits to identify the buffer conditions that will yield protein crystals of WD4-HAH1. Sparse matrix screening samples a broad range of buffers pre-selected from...
previously known conditions for producing protein crystals for X-ray diffraction studies. Sparse matrix screening facilitates the optimization of the buffer conditions, either by changing its composition, or by altering the pH to improve the protein to crystallization conditions. The HR2-110 crystal screen produced crystals with the buffer conditions of 0.2 M sodium citrate tribasic dihydrate, 0.1 M HEPES, NaCl, pH 7.5, 20% (v/v) 2-propanol and 0.2 M zinc acetate dihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, 18% (w/v) polyethylene glycol 8,000. Crystals were also obtained with the HR2-112 crystal screen with buffer conditions of 0.1 M sodium chloride, 0.1 M BICINE pH 9.0, and 20% (v/v) polyethylene glycol monomethyl ether 550. Protein crystals began to form after one week and stopped growing in the second week (Figure 3.4 a and b). The crystals were confirmed to be protein crystals by observing them under a UV microscope (Figure 3.87 c).

Figure 3.11 Protein crystals as viewed under optical microscope. (A) and (B) protein crystals grown with Hamptons crystal screen HR2-110, (C) Crystals as seen under UV microscope, (D) crystals growing on a horse hair, (E) crystals grown using seeds obtained with crystals grown from horse hair, (F) single hexagonal protein crystal.
Optimizing the primary conditions did not improve the quality of the protein crystals obtained. This prompted the use of nucleating agents such as horse hair (Figure 3.4 d) and microseeding techniques that are known to influence nucleation with considerable protein crystallization success.\textsuperscript{22,23,24} Crystals from the initial screening were used as seeds to grow new protein crystals (Figure 3.87 b), some of which grew in different buffer conditions (0.5 M ammonium sulfate, 0.1 M MES monohydrate pH 6.5, 30% polyethylene glycol monomethyl ether 5000). In order to eliminate false positives, the protein crystals were visualized using a UV-light microscope to distinguish them from salt crystals. Proteins with aromatic amino acids are intrinsically fluorescent when excited with UV light. Tryptophan is more fluorescent than tyrosine and phenylalanine.\textsuperscript{25} When a crystal is observed under visible light but not with UV illumination, it is likely a salt crystal.\textsuperscript{25} Crystals were transferred into a cryoprotectant that consisted of the reservoir solution plus 25% (v/v) glycerol and soaked for one minute. Crystals were looped, flash-frozen in liquid nitrogen and sent for X-ray diffraction at the Advanced Photon Source, Argonne National Laboratory, in collaboration with Dr. Amy Rosenzweig of Northwestern University. I would like to especially thank Dr. Ingrid Span and Dr. Laura Dassama who helped mount the crystals. The crystals failed to diffract and data couldn’t be collected for structural determination but promising leads were identified.
3.9 Summary

This chapter covers the materials and methods used for the expression, purification and preliminary crystallization of WD4-HAH1 chimera. The protein was able to crystalize at room temperature with the optimum conditions of 0.5 M ammonium sulfate, 0.1 M MES monohydrate pH 6.5, 30% polyethylene glycol monomethyl ether 5000, giving a single hexagonal crystal (Figure 3.87 f). 25% glycerol was used as a cryoprotectant. Since cryoprotecting crystals also requires optimization, future experiments could involve screening different cryoprotectants or crystalizing the protein under different temperatures.
3.10 REFERENCES


4. Banci, L.; Bertini, I.; Del Conte, R.; D’Onofrio, M.; Rosato, A., Solution structure and backbone dynamics of the Cu(I) and apo forms of the second metal-binding domain of the Menkes protein ATP7A. Biochemistry 2004, 43 (12), 3396-403.


CHAPTER 4

STRUCTURAL BIOINFORMATICS AND EXPERIMENTAL METHODS FOR

PfCuP-ATPASE

4.1 Introduction

PfCuP-ATPase gene (PF3D7_0904900) is located in chromosome 9 of the Plasmodium falciparum genome. It has 7707 bp and encodes a 2568 amino acid protein with a predicted molecular weight of 299390.12 Da. PfCuP-ATPase is the largest protein in the P-ATPase family. The protein is homologous to the human copper ATPases: Menkes (ATP7A) and Wilson disease (ATP7B) proteins. Unlike ATP7A and ATP7B, PfCuP-ATPase has repeated inserts of 40-60 amino acids residues every 100-200 amino acids starting at residue 600.\(^1\) The N-terminal region has only one copper binding motif (MTCDNC). ATP7A and ATP7B have six metal binding domains with MXCXXC motifs and eight TM domains, and CPC motif in the TM region which helps in the heavy metal transduction across the membrane. TM domains were predicted using TMHMM, TopPred2 and TMpred membrane prediction programs. Protein sequence alignment with M. musculus, H. sapiens (ATP7B), S. aureus, P. yoelii, and P. chalaudi orthologues was done using ClustalW2. PfCuP-ATPase, like other P-type ATPases, also has a DKTGT motif that is involved in phosphorylation and a GDGINDCF motif that is involved in the binding of ATP. Between the first and the second predicted TM domains, there is a predicted acidic domain with repeats of 11 QDVKDD motifs (residue 1248-1361). These inserts are not present in other known copper P-type ATPases.
4.2 Structural prediction of PfCuP-ATPase

NCBI BLAST was used to search for sequence homology of the PfCuP-ATPase with the known P-type ATPases. Sequence alignment was done using ClustalW2. ClustalW2 is molecular biology program used for multiple protein sequence alignment. Alignment can be across the entire sequence or only in certain regions. This makes it possible to identify conserved regions of a sequence that may give information that helps in predicting the function and structure of specific proteins.

Structured regions of the PfCuP-ATPase protein sequence were identified using Jpred3, a protein secondary structure prediction server. It uses Jnet algorithms to identify the α-helix, β-strand and coil regions in a protein sequence. Figure 4.0 shows a representative secondary structure prediction of regions of the PfCuP-ATPase protein. The regions of protein sequence that were predicted to contain secondary structures were used to predict the tertiary structures using the Phyre2 server. Phyre2 is a tertiary structure prediction program. Phyre2 is an online tool for the prediction of a potential three dimensional structure of a protein based on the alignment to known protein structures in PDB. The location and orientation of transmembrane helices PfCuP-ATPase were predicted using a hidden Markov model (TMHMM). The topology of PfCuP-ATPase was predicted with TopPred2, an online server for the prediction of hydrophobicity analysis topology prediction of membrane proteins. The hydrophobicity plot (Figure 4.1) suggests that PfCuP-ATPase has ten transmembrane domains.

The portion of the PfCuP-ATPase N-terminus to the first predicted transmembrane domain is about 570 residues. Between the first and the second predicted transmembrane domains
there is a long cytosolic loop (residues 590 - 1500, Figure 4.0). Within this loop there is a predicted hypothetical acidic domain, (residues 1251-1510, Figure 4.3).

4.2.1 N-terminal domains of PfCuP-ATPase

Based on the aforementioned bioinformatics studies, the N-terminal 600 residues of PfCuP-ATPase are predicted to have three structured domains. A long unfolded region exists between domain 1 and domain 2. Domain two and 3 are in close proximity, with a short linker between them. Only domain 2 has the MTCXXC copper binding motif. It was predicted that PfCuP-ATPase has ten transmembrane domains (Figure 4.1). N-terminal domains 2 and 3 are reminiscent of domains 5 and 6 of the Wilson disease protein. The secondary structure of the hypothetical structured region in the N-terminal domain was predicted using Jpred3, and the three-dimensional tertiary structure was predicted using Phyre2 (Figure 4.0 and 4.2).

Figure 4.0 Protein sequence and secondary structure prediction by Jpred3 of domain 1 (residues 1 - 68), domains 2 (residues 390 - 474) and domain 3 (residues 480 – 561).
4.2.2 Transmembrane prediction of \( PfCuP\)-ATPase

Figure 4.1 Hydrophobicity plot for the transmembrane prediction of \( PfCuP\)-ATPase. The transmembrane domains are shown in red bars.
The first predicted N-terminal domain spans residues 1-68 (here in referred to as PfCuP-D1) based on secondary structure prediction using Jpred3 (Figure 3.1) and tertiary structure prediction using phyre2 (Figure 4.2). Phyre2 predicted the hypothetical domain 1 to have a ferredoxin fold with 82% confidence and 21% sequence identity with a heavy metal binding domain of HMA7 (PDB: 3DXS), a copper P-type ATPase. PfCuP-D1 lacks the metal binding motif.

The second predicted domain spans the residues 392–474 (Figure 4.0), is named PfCuP-MBD2, and contains the metal binding motif MTCDNC. This domain is in close proximity to the third hypothetical domain, PfCu-D3. Domain 3 is composed of residues 480–561 and lacks the metal binding motif. The predicted Domain 2 and Domain 3 are separated by a short ten residue linker, and both are predicted to have a ferredoxin fold. They both assume a ferredoxin fold (Figure 4.22). Their structural organization is reminiscent of ATP7B domains 5 and 6 (PDB: 2EW9).
4.2.3 Beta barrel like domain with unknown function

A region of PfCuP-ATPase spanning from residue number 1248 to 1504 contains 11 repeats of the motif QDVKDD. This region is predicted to be structured and contains a hypothetical domain with unknown function (Figure 4.23).

![Figure 4.4 Hypothetical domain with a QDVKDD motif. The domain is predicted to contain mostly beta sheets.]

4.3 Plasmids construction, protein expression and purification of the N-terminal domains of PfCuP-ATPase

This section describes the experimental methods for cloning, protein expression and purification of N-terminal domains of PfCuP-ATPase.

4.3.1 PfCuP-D1 plasmid construction, protein expression and purification

The gene for the Plasmodium falciparum copper ATPase has a high percentage of A and T nucleotides which makes expression in E. coli challenging. A gene construct containing the sequence that encodes the predicted Domain 1 was synthesized by GeneScript, Inc. Piscataway, New Jersey. Codon optimization was used to reduce the A and T content, and keeping the expressed protein sequence unaltered. The synthesized gene was amplified by PCR using KOD Hot Start DNA polymerase (Novagen). Mutagenic primers were designed such that the forward primer introduces NcoI recognition site at the N-terminus of the
*PfCuP-D1* gene while the anti-sense primer incorporates the BamHI site at the C-terminal portion of the protein. The PCR reaction for the *PfCuP-D1* was set up as detailed in Table 4.1

**Table 4.1 *PfCuP-D1* PCR reaction**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Final concentration</th>
<th>PCR Thermo cycler condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-primer</td>
<td>1.5</td>
<td>0.3 µM</td>
<td>Step</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Temperature(°C)</td>
</tr>
<tr>
<td>3'-primer</td>
<td>1.5</td>
<td>0.3 µM</td>
<td>1. Polymerase activation</td>
</tr>
<tr>
<td>2mM dNTPs</td>
<td>5</td>
<td>0.2 mM</td>
<td>2. Deactivation</td>
</tr>
<tr>
<td>MgSO4</td>
<td>3</td>
<td>1.5 mM</td>
<td>3. Annealing</td>
</tr>
<tr>
<td>10X KOD buffer</td>
<td>5</td>
<td>1X</td>
<td>4. Extension</td>
</tr>
<tr>
<td>Template for <em>PfCuP-D1</em></td>
<td>1</td>
<td>120 ng</td>
<td>Repeat steps 2-4 (30 times)</td>
</tr>
<tr>
<td>KOD Hot start DNA polymerase</td>
<td>1</td>
<td>0.02 U/µL</td>
<td>Hold</td>
</tr>
<tr>
<td>Sterile water</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR product for *PfCuP-D1* (Figure 4.31 a) was purified using gel extraction kit from Qiagen. Pure insert DNA and vector DNA were digested with the same restriction endonucleases NcoI and BamHI in order to create the sticky ends that enable the ligation of the gene insert into the pET24d(+) expression vector pET-24d(+). The digested insert is...
225 bp while the digested pET24d(+) vector is 5307 bp. The insert and vector DNA were treated with T4 DNA ligase using the Quick Ligation Kit (BioLabs Inc.) and incubated at room temperature for 20 min. The ligase was deactivated by incubating the reaction mixture at 70°C for 10 min. The vector to insert ratio used for the ligation was 1:3. 1 µL of the ligation reaction was used to transform GigaSingles™ NovaBlue competent cells and colonies grown overnight at 37°C. A single colony was inoculated into 5 mL LB containing 50 µg/mL of kanamycin and grown overnight at 37°C. Single colonies were screened for the successful clones. The plasmids were purified using Qiagen plasmid purification kit. The presence of the insert was confirmed by restriction mapping and agarose gel electrophoresis (Figure 4.31 b and c). Successful clones were further confirmed by DNA sequencing at GeneScript USA Inc. Piscataway, New Jersey.

Figure 4.5PRC reaction and cloning of P/CuP-D1. (a)M; 1 kb DNA marker, 1; purified P/CuP-D1 PCR product. (b) Ligation of P/CuP-D1 into pET24d(+). M; DNA marker, 1; linearized pET24d(+), 2-5; purified pET24d(+) with P/CuP-D1 insert DNA. (c) pET24d(+)-P/CuP-D1 plasmids were digested with Ncol and BamHI. M; DNA marker, 1-4; unsuccessful clones, 5 & 9; plasmids with insert DNA, 6-8; plasmids that did not contain the insert DNA. Samples 5 & 9 were sequenced at GenScript USA Inc. to confirm the clones.
4.3.2 Expression and purification of PfCuP-D1

PfCuP-D1 protein was expressed in *E. coli* BL21(DE3) cells were transformed with the pET24-PfCuP-D1 plasmid and colonies grown at 37°C for 16 hours. A single colony was used to inoculate 5 mL of starter culture (LB media) containing 50 µg/ml Kanamycin sulfate. This starter culture was grown to density in a 37°C shaker and inoculated into a 1 L culture. The culture was induced with a final concentration of 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) when the optical density at 600 nm reached 0.6-0.8. Protein induction was carried out for 4 hours at 37°C, and cells were harvested by centrifugation. Pelleted cells were re-suspended in a lysis buffer – 50 mM 2-(N-morpholino) ethane sulfonic acid (MES), 150 mM sodium chloride, pH 6.5. The supernatant containing soluble proteins was separated from cell debris by centrifugation. The supernatant was then filtered using a 0.22 µm pore size hydrophilic membrane. The protein was purified in a two-step process using cation exchange and size exclusion chromatography. The filtered supernatant was loaded onto a cation exchange column pre-equilibrated with buffer A (50 mM MES buffer, 150 mM NaCl, pH 6.5. The column was washed with three column volumes of buffer A and the protein eluted using a gradient (Figure 4.32a) with buffer B (50 mM MES buffer, 500 mM NaCl). Fractions containing PfCuP-MBD1 protein were pooled together and concentrated using a 50 mL Amicon device fitted with a 3 kDa membrane. The protein sample was then purified further by size exclusion chromatography, using a Superdex 75 High load 26/60 column (Figure 4.32b). The purity of the protein was assessed by SDS-PAGE (Figure 4.33 a and b)
Figure 4.6.
Cation exchange purification of \(PfCuP-D1\).

Figure 4.7
Size exclusion chromatography of \(PfCuP-D1\)

Figure 4.8 (a). Protein expression of \(PfCuP-MBD1\) at 37 °C for 4 hours. 
M; protein marker, 1; 4hrs induction, 2; 3 hrs. induction, 3; 2 hrs. induction, 4;1-hour induction. 
(b) 1; cation exchange fraction, 2; gel filtration fraction.
4.3.3 *PfCuP-MBD2* and *PfCuP-MBD2-3* plasmid construction

*PfCuP-MBD2* is the second N-terminal domain of *PfCuP-ATPase*. It is the only domain with the MXCXXC copper binding motif. The gene encoding *PfCuP-MBD2* was amplified from the synthesized gene *PfCuP-MBD2-3* which was originally cloned into pUC57 by GenScript, Inc. Difficulty of expression and solubilization led to the testing of multiple constructs. These constructs are listed in Table 4.2.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET24d+ <em>PfCuP-MBD2</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression without a tag</td>
</tr>
<tr>
<td>pET24d+ <em>PfCuP-MBD2-N</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression with an N-terminal Histidine tag</td>
</tr>
<tr>
<td>pET24d+ <em>PfCuP-MBD2-C</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression with a C-terminal Histidine tag</td>
</tr>
<tr>
<td>pET32-TEV- <em>PfCuP-MBD2</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression with a thioredoxin fusion tag and a TEV protease site.</td>
</tr>
<tr>
<td>pET32-TEV- <em>PfCuP-MBD2-G</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression with a thioredoxin fusion tag and a poly glycine tag after the TEV protease site.</td>
</tr>
<tr>
<td>pET32-TEV- <em>PfCuP-MBD2-V</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression with a thioredoxin fusion tag and a TEV protease site with a Valine in the second position.</td>
</tr>
<tr>
<td>pET32-TEV- <em>PfCuP-MBD2-GT</em></td>
<td>Plasmid for <em>PfCuP-MBD2</em> expression with a thioredoxin fusion tag and a TEV protease site with a Threonine in the second position.</td>
</tr>
</tbody>
</table>

pET24d+ *PfCuP-MBD2-V* construct was made to express the *PfCuP-MBD2* protein without a purification tag, while pET24d+ *PfCuP-MBD2-N* and pET24d+ *PfCuP-MBD2-
C were designed such that the protein is expressed with a poly histidine tag at the N or C terminus of the proteins, respectively. The PCR reaction for the amplification of PfCuP-MBD2 and PfCuP-MBD2-3 genes was designed such that the forward primers introduce a Neol site at the N-terminal end of the gene while the reverse primer introduces a BamHI recognition site at the C-terminal end. The PCR reaction and the thermocycle conditions are outlined in Table 4.3.

Table 4.3 PCR reaction for PfCuP-MBD2 and PfCuP-MBD2-3

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Final concentration</th>
<th>Thermo cycler conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ primer</td>
<td>1.5</td>
<td>0.3 µM</td>
<td>Step</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time (°C)</td>
</tr>
<tr>
<td>3’ primer</td>
<td>1.5</td>
<td>0.3 µM</td>
<td>1. Polymerase activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>2mM dNTPs</td>
<td>5</td>
<td>0.2 mM</td>
<td>2. Deactivation</td>
</tr>
<tr>
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<td></td>
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<td>94</td>
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<td></td>
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<td>15</td>
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<td>MgSO4</td>
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<td>3. Annealing</td>
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<td></td>
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<td>60</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
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<td>10X KOD buffer</td>
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<td>1X</td>
<td>4. Extension</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>5 s (for MBD2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 s (for MBD2-3)</td>
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<td>Template for PfCuP-MBD2-3</td>
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<td>Repeat steps 2-4, 30 cycles</td>
</tr>
<tr>
<td>KOD Hot start DNA polymerase</td>
<td>1</td>
<td>(0.02 U/µL)</td>
<td>Hold</td>
</tr>
<tr>
<td>Sterile water</td>
<td>32.0</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50.0</strong></td>
<td></td>
<td><strong>∞</strong></td>
</tr>
</tbody>
</table>
The PCR reaction for the amplified *PfCuP-MBD2* gene gave a product without side reactions (Figure 4.34 a). The *PfCuP-MBD2-3* PCR product was purified by gel extraction kit from Qiagen to remove the unwanted products. The *PfCuP-MBD2* PCR product was purified using the Qiagen PCR purification kit protocol. The purified products and the vector DNA were then digested with NcoI and BamHI enzymes and quantified using a 0.8% agarose gel. The *PfCuP-MBD2* digested insert is 303 bp while the digested *PfCuP-MBD2-3* insert is 516 bp (Figure 4.9 a). Linearized pET24d (+) vector is 5263bp. 20 ng of *PfCuP-MBD2* and 35 ng of *PfCuP-MBD2-3* of insert DNA were separately mixed with 120 ng of digested vector DNA and treated with T4 DNA ligase. The ligation mixture was incubated at room temperature for 20 min. The ligase was deactivated by incubating the reaction mixture at 70°C for 10 min. 1 µL of the ligation reaction was then used to transform GigaSingles™ NovaBlue competent cells and incubated overnight at 37°C. A single colony was inoculated into 5 ml LB medium containing 50 µg/ml of kanamycin and grown overnight at 37°C. Single colonies were screened for the successful clones. The plasmids were purified using Qiagen
plasmid purification kit protocol (Figure 4.35 b and c). Successful clones were further confirmed by DNA sequencing at Retrogen, Inc. (San Diego, CA).

**4.3.4 Protein expression and solubility of \( PfCuP-MBD2 \) and \( PfCuP-MBD2-3 \)**

\( PfMBD2 \) and \( PfMBD2-3 \) were expressed by transforming BL21(DE3) cells with the expression plasmid pET24d-\( PfCuP-MBD2 \) and pET24d-\( PfCuP-MBD2-3 \), respectively. Single colonies were used to inoculate into an LB culture with kanamycin sulfate (50 \( \mu \)g/mL final concentration). The culture was induced with a final concentration of 1mM IPTG when the optical density at 600 nm reached 0.6-0.8. The proteins were expressed at 37°C for 3-4 hours and cells harvested by centrifugation. Initial extraction of the protein either by a freeze thaw method or sonication was unsuccessful (Figures 4.10 and 4.11).

![Figure 4.10](image1.png)  
**Figure 4.10** (a) \( PfCuP-MBD2 \) solubility screening, M; marker, 1; extract 1 (freeze thaw), 2; extract 2 (sonication), post extract pellet. Figure 3.36 (b) \( PfCuP-MBD2 \) solubility screening, Pellet, extract 1 (freeze thaw), extract 2 (sonication), extract 3 (cation exchange), post extraction pellet, extraction with 1M urea, extraction with 3M urea, Marker

![Figure 4.11](image2.png)  
**Figure 4.11** \( PfCuP-MBD2-3 \) protein expression and solubility screening. (a) \( PfCuP-MBD2-3 \) protein expressed at 37°C. M; Protein marker, 1; extract by freeze thaw, 2; extract by sonication, 3; pellet before extraction, 4; pellet after extraction. (b) \( PfCuP-MBD2-3 \) protein expression at room temperature (2-5hrs). (c) \( PfCuP-MBD2-3 \) protein extraction. M; marker, 1; pellet before extraction, 2; pellet after extraction, 3; extract by sonication
Both the proteins were formed inclusion bodies when induced at 37°C. Expressing the proteins at a lower temperature (23-30°C) did not increase solubility (Figure 4.11 a, b and c). Different lysis buffer conditions were screened. Extraction by sonication with 50 mM Tris HCl, 150 mM NaCl, pH 8.5 increased the amount of protein in the soluble fraction but not enough for characterization. The proteins were extracted under denaturing conditions with a buffer containing 3M urea (Figure 4.10 b lane 6 and 7). These constructs were abandoned and methods of expression of the protein as a fusion tag were explored. The PfCuP-MBD2 and PfCuP-MBD2-3 were thus re-cloned into pET32Xa/LIC vector, Novagen. The thioredoxin fusion tag is positioned at the N-terminal end of the protein of interest. The tag will be removed by digesting the protein with TEV protease.

4.3.5 Construction of pET32-TEV- PfCuP-MBD2 and pET32-TEV- PfCuP-MBD2-3 plasmid

PfCuP-MBD2 was amplified from the pUC57-PfCuP-MBD2-3 plasmid which was purchased from GeneScript, Inc. Amplification was done using the KOD Hot Start DNA polymerase (Novagen) protocol (Table 4.4and 4.5). The forward primer was designed to include the Tobacco Etch Virus (TEV) protease cleavage site before the start of the PfCuP-MBD2 protein. Both sense and antisense primers contain A sequence compatible with the Ligation Independent Cloning (LIC) cloning into the pETXa/LIC vector. LIC is a directional cloning method of the PCR product without the use of restriction enzymes or ligation reactions.
Table 4.4 KOD Hot Start DNA polymerase protocol (*PfCuP-MBD2 amplification*).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Final concentration</th>
<th>PCR Thermo cycler condition</th>
</tr>
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<tbody>
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<td>5’ primer</td>
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<td>0.3 µM</td>
<td>Step</td>
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<td>Temperature (°C)</td>
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<td></td>
<td>Time(S)</td>
</tr>
<tr>
<td>3’ primer</td>
<td>1.4</td>
<td>0.3 µM</td>
<td>1. Polymerase activation</td>
</tr>
<tr>
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<td>2mM dNTPs</td>
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<td>2. Deactivation</td>
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<td>MgSO4</td>
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<td>1.5 mM</td>
<td>3. Annealing</td>
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<tr>
<td>Template for <em>PfCuP-D1</em></td>
<td>1</td>
<td>120 ng</td>
<td>Repeat steps 2-4, 30 cycles</td>
</tr>
<tr>
<td>KOD Hot start DNA polymerase</td>
<td>1</td>
<td>(0.02 U/µL)</td>
<td>Hold</td>
</tr>
<tr>
<td>Sterile water</td>
<td>32.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.5 KOD Hot Start DNA polymerase protocol (PfCuP-MBD2-3 amplification).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Final concentration</th>
<th>PCR Thermo cycler condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ primer</td>
<td>1.4</td>
<td>0.3µM</td>
<td>Step</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Temperature (°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time (S)</td>
</tr>
<tr>
<td>3’ primer</td>
<td>1.4</td>
<td>0.3 µM</td>
<td>1. Polymerase activation</td>
</tr>
<tr>
<td>2mM dNTPs</td>
<td>5</td>
<td>0.2 mM</td>
<td>2. Deactivation</td>
</tr>
<tr>
<td>MgSO4</td>
<td>3</td>
<td>1.5mM</td>
<td>3. Annealing</td>
</tr>
<tr>
<td>10X KOD buffer</td>
<td>5</td>
<td>1X</td>
<td>4. Extension</td>
</tr>
<tr>
<td>Template for PfCuP-D1</td>
<td>1</td>
<td>120 ng</td>
<td>Repeat steps 2-4, 30 cycles</td>
</tr>
<tr>
<td>KOD Hot start DNA polymerase</td>
<td>1</td>
<td>(0.02 U/µL)</td>
<td>Hold</td>
</tr>
<tr>
<td>Sterile water</td>
<td>32.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The PCR products corresponding to the \textit{PfCuP-MBD2} (334 bp) and \textit{PfCuP-MBD2-3} (545 bp) genes were purified using the PCR purification kit protocol (Qiagen) and quantified using agarose gel electrophoresis (Figure 4.12 a and b).

![Figure 4.12 (A) Purified PCR product for \textit{PfCuP-MBD2} (334 bp) and (B) for \textit{PfCuP-MBD2-3} (545 bp)](image)

### 4.3.6 LIC cloning for \textit{PfCuP-MBD2} and \textit{PfCu-PMBD2-3}

The insert DNA (0.2 pmol) was treated with T4 DNA polymerase as described in Table 4.37. The solution then was mixed and incubated at 22°C for 30 min. The polymerase was subsequently deactivated at 75°C for 20 min. 2 µL of T4 DNA polymerase treated PCR insert was then ligated into 1 µL of pET32Xa/LIC vector at 22°C for 5 min. 1 µL of 25 mM EDTA was added and incubated for a further 5 min at 22°C. 1 µL of the ligation reaction was used to transform GigaSingles\textsuperscript{TM} NovaBlue competent cells, cells were plated and incubated overnight at 37°C. Single colonies were used to inoculate 5 mL of LB with 50 µg/mL of kanamycin and incubated for 16 hours at 37°C. Cells were harvested by centrifugation and the plasmids were purified with the Qiagen plasmid DNA purification kit (Figure 4.13 and 4.14). DNA sequencing at Retrogen, Inc. (San Diego) confirmed
successful clones.

Table 4.6 T4 DNA polymerase protocol (treatment of PCR insert)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 pmol of insert DNA</td>
<td>1</td>
</tr>
<tr>
<td>10 X T4 DNA polymerase buffer</td>
<td>2</td>
</tr>
<tr>
<td>25 mM dGTP</td>
<td>2</td>
</tr>
<tr>
<td>100 mM DTT</td>
<td>1</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td>0.4</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>13.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20.0</strong></td>
</tr>
</tbody>
</table>

4.3.7 Expression and purification of PfCuP-MBD2-Trx and PfCuP-MBD2-3-TRX

PfCuP-MBD2-Trx and PfCuP-MBD2-3-Trx (MBD2 and MBD2-3 with a thioredoxin fusion tag) were expressed by transforming BL21(DE3) competent cells with pET32-PfCuP-MBD2 and pET32-PfCuP-MBD2-3 plasmids, respectively. Single colonies were
inoculated into 5 mL of LB starter culture with 100 µg/mL of ampicillin. When the culture had grown to density it was scaled up to 1L. The culture was induced with 1mM IPTG when the optical density at 600 nm attained 0.6 - 0.8. Proteins were expressed at 37°C for 3 hours (Figure 3.39a) and the cells were harvested by centrifugation. Cells were re-suspended in 50 mM sodium phosphate buffer, 150 mM NaCl, 0.5 mM DTT, pH 7.5. The *Pf*CuP-MBD2-Trx and *Pf*CuP-MBD2-Trx proteins were extracted by sonication (sonication time: 5 min, pulse 15 sec, hold 59 sec and amplitude 40%). The clarified supernatant was obtained by centrifuging the lysate at 15000 rpm, for 20 min at 4°C. The supernatants containing the soluble proteins were filtered through a 0.22 µm pore size membrane and loaded into a HisPrep FF 16/10 nickel column, which has a high affinity for polyhistidine tagged proteins. The column was washed with three column volumes of 50 mM sodium phosphate buffer, 150 mM NaCl, 20 mM imidazole, pH 7.5. The protein was eluted using a gradient 50 mM sodium phosphate, 150 mM NaCl, 300 mM imidazole, pH 7.5. The eluted protein was buffer exchanged on a PD10 desalting column with a TEV cleavage buffer (50 mM Tris/HCl, pH 8.0). To remove the thioredoxin fusion tag, TEV protease was added to the desalted protein (1: 80 mass ratio) and incubated overnight at room temperature. The degree of TEV cleavage was assessed by SDS-PAGE (Figure 4.16 and 4.17).

4.3.8 Inclusion of a fusion tag increased solubility but reduced the TEV protease activity

The attachment of the fusion tag to the *Pf*CuPMBD2 protein increased the solubility of the proteins (Figure 4.39 b). However, it was extremely challenging to remove the fusion tag
using the protease. The final protein sample obtained was not usable for any characterization as much of the protein retained the tag (Figure 4.17)

Figure 4.15. 12.5% SDS-PAGE gel showing the expression of *PfCuP-MBD2Trx* and *PfCuP-MBD2-3Trx*

Figure 4.16. 12.5% SDS-PAGE showing purification of *PfCuP-MBD2*. TEV protease was not able to cut the fusion tag.

Figure 4.17. 12.5% SDS-PAGE gel showing the steps for the purification of *PfCuP-MBD2*. Digestion with TEV protease was not successful. Only about ten percent of the protein was obtained upon tag removal. The activity was at minimal. Optimizing the digestion conditions like temperature, buffer and salt conditions did not increase the activity of the protease.

4.3.9 Addition of polyglycine (six glycine residues) at position 2 of TEV protease recognition site.

Different residues were added at position 2 (P2) after the TEV protease cut site. Addition
of polygycine residues at this position was expected to expose the protease cleavage site making it more accessible to the enzyme. Other residues were inserted into other constructs (two glycine, two valine and two threonine residues) by site directed mutagenesis. All these efforts proved unsuccessful (Figure 4.18).

4.4.0 PfCuP-MBD2-C and PfCuP-MBD2-3 C-terminal poly histidine tag

PfCuP-MBD2 and PfCuP-MBD2-3 were recloned into a pET24d(+) vector that places the TEV protease site and a polyhistidine tag (HHHHHHH) at the C-terminal end of PfCuP-MBD2 (shown in red in Figure 4.41 (b)) and PfCuP-MBD2-3.

The new constructs were named pET24d-PfCuP-MBD2-C and pET24d-PfCuP-MBD2-3-C. A general procedure for the PCR reaction and cloning was followed, as described
previously. Successful clones were confirmed by DNA sequencing.

4.4.1 Expression and purification of PfCuP-MBD2-C and PfCuP-MBD2-3 C-terminal poly histidine tagged proteins

Protein expression was performed at room temperature and at 18°C. The solubility was the highest when the proteins were expressed 18°C. Purification was performed using immobilized nickel ion affinity chromatography (Figure 4.20).

Figure 4.20 His Trap purification of PfCuP-MBD2 and PfCuP-MBD2-3

Figure 4.21 SDS gel for Purification of PfCuP-MBD2. M: protein marker, 1; HisTrap sample, 2; MBD2 with TEV protease, 3; MBD2 after TEV protease digestion

Figure 4.22 Gel filtration Purification of PfCuP-MBD2

Figure 4.23 SDS gel for Purification of PfCuP-MBD2-3. M: protein marker, 1-3; HisTrap sample, 4-5; MBD2-3 with TEV protease, 6; pure MBD2-3 after TEV protease digestion
The histidine tag was removed by digesting the protein with the TEV protease at a mass ratio of 1:80 overnight at room temperature. Pure protein without the affinity tag was collected as a flow through, concentrated and purified further by gel filtration (Figure 4.22). Purity of the protein sample was assessed by SDS-PAGE (Figure 4.21 and 4.23).

4.5.0 *Pf*CuP-D3 construct

N-terminal Domain 3 is closest to the first transmembrane domain in the *Pf*CuP-ATPase. This hypothetical domain lacks the copper binding motif of copper ATPases. The gene sequence spanning this region of the protein was amplified by PCR from the GeneScript Inc. synthesized pUC57-*Pf*CuP-MBD2-3 plasmid. The *Pf*CuP-MBD3 construct amplified did not incorporate the fusion fusion or affinity tags. The forward primer and the reverse primers incorporated NcoI and BamHI cut sites, respectively. The PCR conditions are as outlined in Table 4.7.
Table 4.7 KOD Hot Start DNA polymerase protocol for *PfCuP*-D3 amplification

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Final concentration</th>
<th>Thermo cycler conditions</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ primer</td>
<td>1.5</td>
<td>0.3 µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’ primer</td>
<td>1.5</td>
<td>0.3 µM</td>
<td></td>
<td>Step 1. Polymerase activation</td>
<td>94</td>
<td>300</td>
</tr>
<tr>
<td>2mM dNTPs</td>
<td>5.0</td>
<td>0.2 mM</td>
<td></td>
<td>Step 2. Deactivation</td>
<td>94</td>
<td>15</td>
</tr>
<tr>
<td>MgSO4</td>
<td>3.0</td>
<td>1.5 mM</td>
<td></td>
<td>Step 3. Annealing</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>10X KOD buffer</td>
<td>5.0</td>
<td>1X</td>
<td></td>
<td>Step 4. Extension</td>
<td>72</td>
<td>30</td>
</tr>
<tr>
<td>Template for <em>PfCuP-MBD2-3</em></td>
<td>1.0</td>
<td>120 ng</td>
<td>Repeat steps 2-4 (30 cycles)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KOD Hot start DNA polymerase</td>
<td>1.0</td>
<td>(0.02 U/µL)</td>
<td>Hold</td>
<td></td>
<td>4</td>
<td>∞</td>
</tr>
<tr>
<td>Sterile water</td>
<td>32.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>50.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The PCR product was purified using the Qiagen gel extraction kit protocol and digested with NcoI and BamHI restriction endonucleases. Cloning of the PfCuP-MBD3 into pET24d+ vector was carried out as previously described in the cloning of PfCuP-D1.

4.5.1 Expression and purification of PfCuP-D3

The expression plasmid for PfCuP-D3 was used to transform BL21 (DE3) competent cells, which were plated overnight at 37°C. A single colony was used to inoculate LB culture with kanamycin sulfate (50µg/ml final concentration) and grown at 37°C. The culture was induced with a final concentration of 1mM IPTG when the optical density at 600 nm reached 0.6-0.8. The proteins were expressed at 37°C for 3 hours and cells harvested by centrifugation. Pelleted cells were re-suspended in lysis buffer: 50 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7.5. The suspension was sonicated (for 5 min, 15 sec pulse, 59 sec hold, and 40% amplitude) and centrifuged for 20 min at 4°C. The supernatant was filtered and loaded onto a anion exchange column (DEAE sepha rose) pre-equilibrated with lysis buffer. The column was washed with three column volumes of the same buffer and
protein was eluted using a gradient with 50 mM sodium phosphate buffer, 1 mM DTT, 500 mM NaCl, pH 7.5 (Figure 4.25). Fractions containing the protein were combined together and purified further using a size exclusion chromatography column, Highload Superdex 75 16/60 (Figure 4.26). Fractions containing pure protein were combined together, concentrated and the purity assessed by SDS-PAGE (Figure 4.27).

![Figure 4.25 Anion exchange purification of PfCuP-D3](image1)

![Figure 4.26 Size exclusion chromatography for PfCuP-D3. The protein purifies both as a monomer and a dimer](image2)

![Figure 4.27 Purification steps for PfCuP-MBD3. 1=marker, 2= pellet, 3= extract, 4= purification by anion exchange, 5=; gel filtration purification](image3)
4.6 Biophysical methods used for characterization of N-terminal domains of PfCuP-ATPase

4.6.1 Circular Dichroism (CD)

CD spectroscopy is used to study chiral molecules of all sizes, and has many applications in biomolecular studies. Its primary use is the determination of relative amounts of secondary structure, and the overall conformation of the macromolecule. The technique is useful in folding and binding properties of proteins.\textsuperscript{12} Secondary structure of biological molecules such as proteins can be effected by pH and temperature. CD can be used to study the structural changes in macromolecules due to changes in environmental conditions, the interactions with other molecules, and kinetic and thermodynamic properties.

CD spectroscopy is measured in the both visible and ultraviolet region of the electromagnetic spectrum. The CD signal can be positive or negative. If the left-handed circularly polarized light (L-CPL) is absorbed to a greater extent than the right-handed circularly polarized light (R-CPL) the signal will be positive. The signal will be negative if the L-CPL is absorbed at a lesser extent than the R-CPL.

4.6.2 CD measurements units and conversions

Circular dichroism is measured as the differential absorbance of the L-CPL and R-CPL. It is expressed as:

\[ CD = \Delta A(\lambda) = A(\lambda)_{L\text{-CPL}} - A(\lambda)_{R\text{-CPL}} \]  \hspace{1cm} \text{Eq. 4.3}

where \( \lambda \) is the wavelength.

Molar circular dichroism

\[ \Delta \epsilon = \epsilon_{L\text{CPL}} - \epsilon_{R\text{CPL}} = \frac{\Delta A}{c \cdot l} \]  \hspace{1cm} \text{Eq. 4.4}
Where \( C \) is concentration in moles/L and \( l \) is the path length in cm

**Mean residue molar circular dichroism (\( \Delta \epsilon_{\text{MR}} \))**

Mean residue molar circular dichroism is a unit that is specific for protein. It measures the molar CD for individual residues instead of the entire protein.

\[
\Delta \epsilon_{\text{MR}} = \frac{\Delta A}{CMR \times l}
\]  
Eq. 4.5

CMR (mean residue concentration) = \( C \times N \)

\( N \) = number of the amino acids in the protein.

If the sequence of the protein is not known, CMR can be calculated using average amino acid weight of 113 Daltons.

\[
\text{CMR} = \frac{p}{113}
\]  
Eq. 4.6

**Degrees of ellipticity (\( \Theta \))**

When linearly polarized light is passed through a chiral sample it will be elliptically polarized i.e. light that is not fully circular polarized (light that is elliptical in shape).

\[
\Delta A = \frac{\Theta}{32.982}
\]  
Eq. 4.7

\( [\Theta] \) is expressed in degrees \( \text{cm}^2 \text{dmol}^{-1} \) or in degrees \( M^{-1} \text{m}^{-1} \)

\[
M^{-1} \text{m}^{-1} = \frac{1000 \text{cm}^3}{\text{mol} \times 100 \text{cm}} = \frac{10 \text{cm}^2}{\text{mol}} = \text{cm}^2 \text{dmol}^{-1}
\]

\[
[\Theta] = \frac{100 \times \Theta}{C \times l}
\]  
Eq. 4.8

\( \Delta \epsilon = \frac{[\Theta]}{3298.2} \) \( \text{the factor is 100 fold larger than the absorbance since the path length for ellipticity if defined in meters.} \)

**Mean residue ellipticity \( ([\Theta]_\text{MR}) \)**

\( [\Theta]_\text{MR} \) is the molar ellipticity of individual residues in a protein.

\[
[\Theta]_\text{MR} = \frac{100 \times \Theta}{CMR \times l}
\]  
Eq. 4.9
4.7 Circular Dichroism of biological molecules

19 out of the 20 amino acids found in proteins are chiral, as are the nucleic acids, DNA and RNA. Therefore, CD is an important tool for studying these biological molecules. The CD spectra of proteins and nucleic acids is influenced by the three dimensional structure of the macromolecule.

4.7.1 Protein circular dichroism

The CD spectra in the far-UV can be used to predict the percentage of each of secondary structural element in a protein, making it an important method to evaluate the secondary structure, folding, binding properties and functional properties of proteins. When the amide chromophores of a polypeptide backbone of a protein are aligned in arrays, the optical transitions reflect multiple transitions resulting in a characteristic CD spectrum that represents the structural elements of the macromolecule. The most studied circular dichroism signatures are the secondary structures of proteins α-helices, β-sheets and random coil. These secondary structures have particular signatures in the circular dichroism spectrum. α-helical proteins have negative bands at 222 nm and 208 nm and a positive band at 193 nm$^{13}$, β-strands have a negative band at 218 nm and a positive band at 195 nm, the random coil (disordered proteins ) have very low ellipticity above 210 nm and negative bands near 195 nm$^{14}$(Figure 4.7). In the near-UV region of the spectra (250-350 nm) the chromophores used for the CD signal are aromatic amino acids and the disulfide bonds of cysteine. Tyrosine gives a signal between 270-290 nm, phenylalanine at 250-270 nm and tryptophan residues at 280-300 nm. Disulfide bonds gives a broad weak
signal that is spread throughout the entire-UV region. Well defined signals in the near UV-region is indicative of a well folded protein.\textsuperscript{15,16}

4.7.2 Nuclear magnetic resonance (NMR) spectroscopy

Understanding the relationship between structure and function of proteins, especially in modern biology, is to determine the three dimensional protein structure. Nuclear magnetic resonance (NMR) spectroscopy is a unique technique that can be used to determine the
protein structure and investigate protein-protein interactions. An advantage of NMR spectroscopy is that the molecules are free in solution, and not restrained by unnatural parking forces. This is particularly important for proteins or complexes that are not easy to crystallize because regions of the protein are conformationally dynamic. The proteins studied are either singly or doubly isotopically labelled with $^{13}$C and $^{15}$N. This is achieved by expressing the protein in minimal media supplemented with labeled carbon and nitrogen sources.

To isotopically label samples, protein expression plasmids were transformed into BL21(DE3) cells and colonies were grown at 37°C overnight on LB/agar plates containing Kanamycin. One colony was used to inoculate a 5 mL starter culture of LB media, which was grown overnight at 37°C with shaking at 250 rpm. 500 µl of the starter culture was then used to inoculate 500 mL of minimal media and protein expression was induced by adding 1 mM IPTG when O. D$_{600}$ = 0.6. $P_f/CuP$-D1 and $P_f/CuP$-D3 were expressed for four hours at 37°C while $P_f/CuP$-D2 and $P_f/CuP$-D2-3 were expressed at room temperature for six hours. 1 L of minimal media contained 42 mM Na$_2$HPO$_4$, 22 mM of KH$_2$PO$_4$, 10 mM NaCl, 11 mM $^{13}$C glucose, 18 mM $^{15}$N NH$_4$Cl, 1 mM MgSO$_4$, 0.02 mM CaCl$_2$, 1 µg thiamine and 50 µg/ml of kanamycin. Protein purification was carried according to the the protocol in Section 4.3.2, 4.4.1and 4.5.1

The concentration of proteins in the NMR samples ranged between 300 µM- 600 µM. The optimal solution for NMT studies contained 30 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl and 1 mM DTT. All experiments were performed at 25°C. All chemical shifts were referenced to a 100 µM of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) standard was used to reference the chemical shifts. A summary of the experiments that
were run on various constructs of *PfCuP*-ATPase are shown in Table 4.8

**Table 4.8 Solution NMR experiments performed on the protein constructs**

<table>
<thead>
<tr>
<th>Protein construct</th>
<th>Experiment</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PfCuP</em>-D1</td>
<td>$^1$H-$^{15}$N HSQC</td>
<td>Shows all H-N correlations</td>
</tr>
<tr>
<td><em>PfCuP</em>-MBD2</td>
<td>$^1$H-$^{15}$N HSQC</td>
<td>Shows all H-N correlations</td>
</tr>
<tr>
<td><em>PfCuP</em>-D3</td>
<td>$^1$H-$^{15}$N HSQC</td>
<td>Shows all H-N correlations</td>
</tr>
<tr>
<td></td>
<td><strong>HNCACB</strong></td>
<td>Correlates the chemical shift of an amide proton to the Cα and Cβ of the same residue and those of the preceding residue.</td>
</tr>
<tr>
<td></td>
<td><strong>HNCA</strong></td>
<td>Correlates the chemical shift of amide proton of a residue the Cα of the same residue as well as the Cα of the preceding residue. Magnetization is transferred from the amide proton to $^{13}$Cα via the N-Cα J-coupling and back to $^{15}$N and $^1$H hydrogen for detection. (^{22,23})</td>
</tr>
<tr>
<td></td>
<td><strong>CBCA(CO)NH</strong></td>
<td>Magnetization is transferred from $^1$Hα and $^1$Hβ to $^{13}$Cα and $^{13}$Cβ, respectively, and then from $^{13}$Cβ to $^{13}$Cα. It is then transferred first to $^{13}$CO, then to $^{15}$NH and then to $^1$HN for detection. (^{24})</td>
</tr>
<tr>
<td></td>
<td><strong>HN(CO)CA</strong></td>
<td>Often used together with HNCA. It correlates the resonances of the amide proton of a residue with the Cα of the preceding residue.</td>
</tr>
<tr>
<td></td>
<td><strong>HNCO</strong></td>
<td>Correlates between the $^{15}$N-$^1$H pair of one residue with the carbonyl ($^{13}$CO) resonance of the preceding residue</td>
</tr>
</tbody>
</table>
Table 4.72 –Continued

| NOESY | Magnetization is exchanged between all hydrogen atoms within 5 Å then transferred to neighboring \(^{15}\)N nuclei and back to \(^1\)H for detection.\(^{25}\) |

4.7.3 \textit{PfCuP-D3} backbone assignments

Backbone resonance assignments for \textit{PfCuP-D3} were obtained by using the \(^1\)H-\(^{15}\)N HSQC, CBCANH and CBCA(CO)NH spectra. The general approach involves the walking the protein backbone and sequentially making chemical shifts assignments using pairs of experiments. For example, the HNCACB shows correlations between N(i), H-N(i) with C\(\alpha\) and C\(\beta\) of i and i-1, while the CBCA(CO)NH spectra shows correlations between N(i), H-N(i) and the C\(\alpha\) and C\(\beta\) atoms of i-1. Using \(^1\)H,\(^{15}\)N HSQC spectra, a single peak was randomly selected and named i. The same peak was then identified in the 3D experiments, and the 1H, 13C planes revealed the associated 13C chemical shifts. The HNCBCA shows carbon resonances, corresponding to the C\(\alpha\) and C\(\beta\) from i-1, and ii residues. Assignment of the four resonances to the i-1 or I residue was achieved by using CBCA(CO)NH which only shows correlations to the C\(\alpha\) and C\(\beta\) from i-1 and i. The two resonances that appear
in the HNCACB experiment, but not in the CBCA(CO)NH experiments are assigned to residue i. The $^{13}\text{C}$ chemical shifts of residue I were compared to those of all resonances in the CBCA(CO)NH experiment to identify the N and H chemical shift of the next residue (i+i) in the protein sequence. These steps were sequentially repeated to ‘walk’ along the backbone of the amino acid sequence, and connecting the assignments for residues i-1, i, i+1, i+2, i+3…i+n. Ascribing the sequentially connected assignments to specific amino acids in the protein sequence makes use of the unique spectral properties of certain amino acids. Serine and Threonine, for instance, have C$\beta$ resonances at higher ppm values (downfield) than C$\alpha$ resonances. Also, Glycine only has a C$\alpha$ atom, Alanine has a C$\beta$ resonance at a low ppm (upfield), and Proline lacks a backbone NH resonance. Figure 4.29 shows representative strip plots from residues 506 to 512.

![Figure 4.29 Backbone assignment between residues 506 – 512. The arrows show the connection of the peaks during the ‘walk’ through in assignments steps.](image-url)
4.7.4 Determination of the Cu\(^{1}\) binding affinities

Experiments to determine the copper binding affinities of PfCuP-MBD2, PfCuP-MBD2-3, DdCuP-MBD1 and DdAtox1 were performed in an anaerobic glove box (NEXXUS chamber in N\(_2\) atmosphere) by reacting apoprotein with Cu\(^{1}\)\([(\text{BCS})_2]\)\(^{3-}\) in deoxygenated buffers. The proteins were reduced excess DTT (≥10 mM DTT) in the glove box, and then the buffer was exchanged into 30 mM sodium phosphate, 150 mM NaCl, pH 7.5, using a PD10 desalting column. The buffer solutions were made fresh and degassed before use. The protein was concentrated using Amicon device with a 3 kDa membrane and the protein concentration was determined using a BCA assay. The copper ligand complex Cu\(^{1}\)\([(\text{BCS})_2]\)\(^{3-}\) was formed by mixing Cu\(^{1}\) in the form of tetrakis(acetonitrile)copper(I) hexafluorophosphate \([(\text{CH}_3\text{CN})_4\text{Cu}]\text{PF}_6\) with Bathocuproinedisulfonic acid (BCS) at a ratio of 1:2.5 to ensure formation of the 1:2 complex, [Cu\(^{1}\)\((\text{BCS})_2\)]\(^{3-}\), with negligible contribution of the 1:1 complex, Cu\(^{1}\)\((\text{BCS})\)\(^{-}\).\(^{26}\) The BCS affinity for copper(I) is comparable to that of protein thiols at subfemtomolar concentrations. It reacts with Cu(I) in 1:2 to form a stable complex anion [Cu\(^{1}\)\((\text{BCS})_2\)]\(^{3-}\) with a maximum absorption at 483 nm.\(^{27}\) A series of reactions were prepared by increasing the apoprotein concentration by titrating it into the complex ligand. The solutions were adjusted with the reaction buffer to a fixed volume so as to keep the concentration of (Cu\(^{1}\)\((\text{BCS})_2\))\(^{3-}\) constant, and only the concentration of protein in all the solutions was varied. The apoproteins were titrated against two different with different concentration of [Cu\(^{1}\)\((\text{BCS})_2\)]\(^{3-}\) complex, with at least two independent replicates. Copper affinity binding constants was measured using the transfer of Cu\(^{1}\) independent replicates. The copper binding affinities was measured using
the transfer of Cu$^1$ from the [Cu$^1$(BCS)$_2$]$^{3-}$ to the protein as established by the change in absorbance for [Cu$^1$(BCS)$_2$]$^{3-}$ at 483 nm.
4.8 REFERENCES


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CHAPTER 5

BIOPHYSICAL PROPERTIES OF THE N-TERMIAL DOMAINS OF \( P_fCuP \)-ATPase: \( P_fCuP-D1, P_fCuP-MBD2, P_fCuP-MBD2-3 \) and \( P_fCuP-D3 \)

5.1 Introduction

Understanding the physical properties of the N-terminal domains of \( P_fCuP \)-ATPase will help elucidate the copper transport mechanism of the protein. The N-terminal region is about 564 amino acid residues in length, yet it has only one copper binding domain. The two additional domains lacking the metal binding motifs had not been previously identified. These domains are connected with a linker of varying lengths. Domain 1 is linked to domain 2 with approximately 321 residues while Domain 2 is linked to Domain 3 by a ten residue linker. \( P_fCuP-D1 \) is the first predicted N-terminal domain of \( P_fCuP \)-PATPase. Since it does not have the copper binding motif, Domain 1 is unlikely to bind the copper(I). However, it is interesting all the three domains are predicted to have a ferredoxin-like fold. Experiments were performed to determine the structural features of these domains.

5.1.1 Biophysical characterization of \( P_fCuP-D1 \)

5.1.2 Circular dichroism

A CD wavelength scan on \( P_fCuP-D1 \) was performed at a 10 \( \mu \)M concentration in a 50 mM sodium phosphate buffer, pH 7.5. Results show that the protein is structured and may contain both alpha helices and beta strands shown in Figure 5.0.
Alpha helix content (% \( \alpha \)) of \( P^f \)CuP-D1

\[
\% \alpha = \frac{MRE_{222}}{X_H^\infty} \times 100
\]

Eq. 5.1

\( MRE_{222} \) is the mean ellipticity at 222 nm and \( X_H^\infty \) is the maximum ellipticity which is determined by the equations:

\[
MRE = \frac{[\Theta]}{\# Res} \quad \text{Eq. 5.2}
\]

\[
[\Theta] = \frac{\theta}{10 \times c \times l}
\]

\[
X_H^\infty = -40000 \times (1 - \frac{2.5}{\# Res}) \quad \text{Eq. 5.3}
\]

Calculations:

\[
[\Theta] = \frac{\theta}{10 \times c \times l} = \frac{-3.66723}{10 + 10^{-6} + 0.1} = -366723 \quad \text{Eq. 5.4}
\]

\[
MRE = \frac{[\Theta]}{\# Res} = \frac{-366723}{74} = -4955.71621 \quad \text{Eq. 5.5}
\]

\[
X_H^\infty = -40000 \times (1 - \frac{2.5}{\# Res}) = -40000 \times (1 - \frac{2.5}{74}) = -38648.648 \quad \text{Eq. 5.6}
\]

\[
\% \alpha = \frac{MRE_{222}}{X_H^\infty} \times 100 = \frac{-4955.71621}{-38648.648} \times 100 = 12.82\% \quad \text{Eq. 5.7}
\]

5.1.3 Chemical unfolding for \( P^f \)CuP-D1

\( P^f \)CuP-MBD1 was found to be relatively unstable. At 2.8±0.2M GuHCl, the amount of denatured protein is equivalent to the amount of the native protein, where \( F_D = F_N \).
(Figure 5.2). A series of samples containing a fixed protein concentration (10 µM) were mixed with guanidine hydrochloride in varying concentration. All samples were adjusted to a fixed volume to keep the protein concentration the same. Only the GuHCl concentration was varied between 0 M and 7.5 M. Samples were equilibrated at room temperature for two hours and the wavelength scan spectra was collected on a JASCO J-815 Spectropolarimeter. The unfolding was monitored at 222 nm, and the CD signal plotted as a fractional denaturation ratio versus guanidine hydrochloride concentration (Figure 5.1).

![Figure 5.1 Chemical unfolding of PfCuP-D1](image)

Chemical unfolding for PfCuP-D1 is sigmoidal and the CD data was fit to a two state model equation 4.0 using Origin graphing analysis software.

\[
S_{obs} = \frac{S_N + S_D \exp\left(-\frac{\Delta G_{H_2O-m[GuHCl]}}{RT}\right)}{1 + \exp\left(-\frac{\Delta G_{H_2O-m[GuHCl]}}{RT}\right)}
\]

Eq. 5.8

\( S_{obs} \) is the signal intensity observed, \( S_N \) is the signal intensity of protein in native state (at 0 M GuHCl), \( S_D \) is the signal of fully denatured protein (at the highest GuHCl concentration). In a two state unfolding, the protein exists either in a native state (N) or in the denatured state (D). At zero concentration of the denaturant, the protein is in native
state. As the concentration of the denaturant increases, the forces stabilizing the protein are gradually disrupted and the protein begins to unfold. The fully denatured state is obtained at the highest concentration of the denaturant. In a two state unfolding, the free energy of unfolding can be determined according to equation 5.9:

\[ \Delta G_D = \Delta G_{H_2O}^\circ - m[GuHCl] \]

\(\Delta G_{H_2O}^\circ\) is the free energy of unfolding at zero concentration of the denaturant and ‘m’ is the slope, which is the measure of the denaturant ability to unfold a protein.\(^2\,3\)

Free energy is related to the equilibrium constant, \(K_{eq}\) which is independent of the protein concentration (Equation 5.10).

\[ \Delta G_D = -RT\ln K_{eq} \]

Keq can be obtained from the fractional denaturation according to equation 5.11

\[ K_{eq} = \frac{F_D}{1-F_D} \]

Where \(F_D\) is given by

\[ F_D = \frac{y_S - y_N}{y_D - y_N} \]
Y_S is the CD signal at a specific GuHCl concentration, Y_N is the CD signal of the native protein and Y_D is the CD signal of the denatured protein.

Free energy of PfCuP-D1 unfolding was determined from a plot of ΔG_D against [GuHCl], according to equation 4.1, which gives a linear relationship. ΔG°_H₂O is obtained from the extrapolation of the curve to the Y axis intercept where [GuHCl] = 0. (Figure 5.2). ΔG°_H₂O for PfCuP-D1 was determined to be 9.0±0.8 kJ/mol. GuHCl concentration where FD = 0.5 was determined to be 2.8±0.2 M (Figure 5.1)

5.1.4 Thermal unfolding for PfCuP-D1

10 μM of PfCuP-D1 was used to measure the thermal unfolding of the protein. The temperature was gradually increased in 2°C/min increments to 95°C. The CD signal was monitored at 222 nm. Figure 5.1 shows a plot of CD signal in molar ellipticity as a function of temperature in °C.

![Figure 5.3 Thermal denaturation of PfCuP-D1.](image_url)
The denaturation of PfCuP-D1 follows a sigmoidal, two state unfolding model. The melting temperature for PfCuP-D1 was determined to be 68.1 ±3.6°C. Thermal free energy of unfolding was determined by plotting lnKeq as a function of T⁻¹ (K⁻¹) according to the van’t Hoff equation 5.14.

\[
\ln \text{Keq} = \frac{\Delta H}{RT} + \frac{\Delta S}{R} \\
\text{Eq. 5.14}
\]

From the Van’t Hoff (equation 4.6) plot, \(\frac{\Delta H}{R}\) is the slope while \(\frac{\Delta S}{R}\) is the y-intercept (Figure 5.4).

![Figure 5.4 Determination of \(\Delta G\) of the thermal denaturation of PfCuP-D1.](image)

\(\Delta G\) was then calculated from equation 4.6 at 298 K

\[
\Delta G = \Delta H - T \Delta S \\
\text{Eq. 5.15}
\]

\(\Delta G\) was determined to be 8.8±1.3 kJmol⁻¹.

### 5.1.5 Biophysical characterization of PfCuP-MBD2

PfCuP-MBD2 is the second domain of PfCuP-ATPase and is the only domain that has a copper binding motif (MXCXXC).
5.1.6 Circular dichroism of \( Pf\)CuP-MBD2

15 \( \mu \)M of \( Pf\)CuP-MBD2 was used for the secondary structure determination of the domain in a 30 mM sodium phosphate buffer, pH 7.5. The protein concentration was determined using BCA assay. Figure 5.16 shows the wavelength scan for \( Pf\)CuP-MBD2 at room temperature.

Figure 5.5 Wavelength scan for \( Pf\)CuP-MBD2. A Minimum at 207nm, 222nm, positive maxima at 197nm indicates a mixture of alpha helix and beta sheets. MBD2 has a 27% alpha helical content.

5.1.7 Chemical unfolding of \( Pf\)CuP-MBD2

\( Pf\)CuP-MBD2 protein samples were buffer exchanged into a 30 mM sodium phosphate, pH 7.5 and the concentration determined using BCA assay. A fixed protein concentration (10 \( \mu \)M) was mixed with varying concentrations of GuHCl, GuHCl was prepared as a stock solution according to the equation 5.16.

\[
C = 57.17 (\Delta N) + 38.68 (\Delta N)^2 - 91.6 (\Delta N)^3
\]

Eq. 5.16

Where C is the concentration of GuHCl in moles/L and \( \Delta N \) is the change in refractive index between the solution and water.
Samples were incubated at room temperature for 2 hours and unfolding was monitored at 222 nm. The CD signal, in molar ellipticity, was plotted as a function of guanidine hydrochloride concentration (Figure 5.17 a). The protein is structurally unstable as it begins to unfold at minimal concentration of GuHCl, and is fully unfolded at 3M GuHCl (Figure 5.6). At 1.35 M GuHCl, $F_D = F_N$, where 50% of the protein is denatured (Figure 5.7).

The denaturation of *PfCuP-MBD2* reveals a two state unfolding. The protein starts to unfold with less than 0.5 M GuHCl and at ~ 1.35 M GuHCl the amount of protein in the
native state is equal to the amount of protein in denatured state. *PfCuP-MBD2* may thus be an unstable protein.

### 5.1.8 Gibbs free energy of chemical denaturation of *PfCuP-MBD2* (*ΔG°* for MBD2 at 298K)

The Gibbs free energy for denaturation of *PfCuP-MBD2* was determined according to equation 5.10. *ΔG°* for *PfCuP-MBD2* was determined to be 6.07 ± 1.10 kJmol⁻¹ (Figure 5.18).

![Figure 5.8 ΔG° for PfCuP-MBD2 at 298K](image)

*ΔG°* was determined by extrapolating the linear curve to Y-intercept where [GuHCl] = 0. The low energy of denaturation suggests the protein is unstable.

### 5.1.9 Heteronuclear Single Quantum Coherence (*¹H, ¹⁵N HSQC*) for *PfCuP-MBD2*

Each amino acid residue in a protein, except proline, has an amide proton that is attached to a nitrogen in a peptide bond. *¹H,¹⁵N HSQC* experiments reveal the chemical shifts of the nitrogen and the amide proton since each N-H bond produces a peak in the spectrum.
The side chain carboximide N-H bonds in asparagine and glutamine residues are also observed in the spectrum, and usually appear as peak doublets in the top right corner of the spectrum (Figure 5.19). If the protein domain is structured the peaks will be well dispersed and are generally easily distinguished from each other. Clusters of overlapped peaks in the middle of the spectrum generally correspond to unstructured residues in the protein. Protein size may also affect the quality of the spectrum, and proteins proteins may not give a good HSQC spectrum. An increase in molecular size causes the molecule to tumble slower, leads to a slower tumbling rate of the molecule, and leads to a rapid relaxation of the excited nuclei and results in the broadening of the associated signals. Proteins smaller than 20 kDa are routinely studied by solution NMR. The collection of a $^1$H, $^{15}$N HSQC NMR spectrum requires the protein to be labeled with $^{15}$N. Proteins constructs were isotopically labelled by expressing them in a media that contains ($^{15}$NH4)$_2$SO$_4$ as the sole nitrogen source. 500 µL of protein was prepared for NMR experiments. Purified PfCuP-MBD2 was buffer exchanged into NMR buffer (30 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7.5) and concentrated to 0.6 mM. Spectra was collected on an 800 MHZ Bruker NMR equipped with cryoprobe at the University of Notre Dame by Professor Blair Szymczyna. The data was processed using NMRPipe software. The PfCuP-MBD2 construct yields a well dispersed $^{15}$N HSQC spectrum, which suggests that Domain 2 and corroborates the circular dichroism result (Figure 5.9).
5.2 Copper metal binding affinity of PfCuP-MBD2

The metal binding affinity of PfCuP-MBD2 and PfCuP-MBD2-3 was determined according to equation 5.17 and 5.18. \(^4,^5\)

\[
P + [\text{Cu}^{1}\text{L}_2]^{3-} \rightleftharpoons \text{Cu}^{1}\text{P} + 2\text{L}^{2-} \tag{5.17}
\]

\[
\frac{[P]_{\text{tot}}}{[\text{Cu}]_{\text{tot}}} = 1 - \frac{[\text{Cu}^{1}\text{L}_2]}{[\text{Cu}]_{\text{tot}}} + K_D \beta_2 \left( \frac{[\text{L}]_{\text{tot}}}{[\text{Cu}^{1}\text{L}_2]} \right)^2 [\text{Cu}^{1}\text{L}_2] \left( \frac{[\text{Cu}^{1}\text{L}_2]}{[\text{Cu}]_{\text{tot}}} \right) \tag{5.18}
\]

Where \(\beta\) is the formation constant for \([\text{Cu}^{1}(\text{BCS})_2]^{3-}\) = \(10^{19.8} \text{ M}^{-2}\)

\(K_D\) is the dissociation constant for the copper-protein complex (Cu\(^{1}\)-P), P is protein, L is the BCS ligand and \(\text{Cu}^{1}\text{L}_2\) is \([\text{Cu}^{1}(\text{BCS})_2]^{3-}\)
Cu¹ and Bathocuproinedisulfonic acid (Na₂BCS) forms a stable 1:2 complex [Cu¹(BCS)₂]³⁻ with the overall formation constant β = 10¹⁹.8 M⁻², as determined by Xiao et al.⁶ The affinity of copper binding to PfCuP-MBD2 was determined by measuring the decrease in absorbance at 483 nm of [Cu¹(BCS)₂]³⁻ (Figure 5.10) and fitting the curve according to equation 5.18 with Origin® (Figure 5.11 and 5.12).

Figure 5.10 A representative spectrum for Absorption spectra of the titration of PfCuP-MBD2 and PfCuP-MBD2-3 against 400 µM BCS. The decrease in absorbance was followed at 483 nm.

Figure 5.11 Titration of PfCuP-MBD2 against 500 µM BCS (black spheres) and 400 µM BCS (red spheres).

Figure 5.12 determination of the dissociation constant k_D by fitting the curve to equation 4.9. k_D was found to be 1.35 x 10⁻¹⁸ M.
5.3 Biophysical characterization of PfCuP-D3

PfCuP-D3 is the third predicted domain in the N-terminal portion of the PfCuP-ATPase. The domain does not contain the copper metal binding motif (MXCXXC). It is closest to the first predicted transmembrane domain and its separated by ten residues from PfCuP-MBD2. This section describes the circular dichroism measurements and triple resonance experiments for PfCuP-D3.

5.3.1 Circular dichroism of PfCuP-D3

Purified PfCuP-MBD3 was buffer exchanged into 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5. 10 µM of the protein in a 1mm quartz cuvette was used for all CD measurements. Wavelength scan spectra was collected between 195 – 300 nm at 25°C and at a scanning rate of 100 nm/min, with three scans in each run. The alpha helical content of PfCuP-D3 was calculated as described in Section 5.1.2 and revealed to be 16%. Figure 5.13 shows the CD spectra for PfCuP-D3.

![Figure 5.13 CD wavelength scan for PfCuP-MBD3. Percentage alpha content was determined to be 16%. The spectra the protein is composed of both alpha helices, and beta strands.](image)
5.3.2 Chemical unfolding of PfCuP-D3 with GuHCl

10 µM of PfCuP-MBD3 was incubated for two hours at room temperature in increasing concentrations of GuHCl. CD signal was recorded at a 222 nm. The unfolding profile of PfCuP-D3 is sigmoidal. The protein begins to unfold at 3 M GuHCl and is completely denatured at 6 M GuHCl (Figure 5.14). The fractional denaturation curve shows that only 50% of the protein is denatured at 4.6 M GuHCl. (FD = FN, Figure 5.15). The Gibbs free energy for the unfolding of PfCuP-D3 was determined to be 15.9 ± 0.8 kJmol⁻¹ at 298 K (Figure 5.16).

Figure 5.14 Chemical unfolding of PfCuP-D3

Figure 5.15. Fractional denaturation of PfCuP-D3

Figure 5.16. Chemical unfolding ΔG for PfCuP-D3 was determined to be 15.9 ± 0.8 kJmol⁻¹ at 298 K
5.3.3 *PfCuP-D3* $^1\text{H}-^{15}\text{N}$ HSQC

$^1\text{H}-^{15}\text{N}$ HSQC is the most common experiment in protein NMR spectroscopy since it shows all the H-N correlations, which mainly are associated with the backbone amide groups. The spectrum is often referred to as a fingerprint of protein domains, and can indicate that other experiments, such as triple resonance spectra, are worth pursuing. It is also useful in assessing protein stability, foldedness, aggregation and the identifying unstructured regions in proteins.\(^7\)

The $^{15}\text{N}$ labelled protein was buffer exchanged into 30 mM sodium phosphate, 150 mM sodium chloride, and 1mM DTT. The protein was concentrated to 0.7 mM, and loaded into a thin walled 1000 MHZ NMR tubed. The NMR data was collected using a Bruker AVANCE II 800 MHz NMR at Notre Dame by Dr. Blair Szymczyna. The data was processed using NMRPipe software. The $^1\text{H},^{15}\text{N}$ HSQC spectrum (Figure 5.17) reveals well dispersed peaks that are characteristic of a folded protein. This result implies the protein construct is a structured domain and may be feasible for triple resonance experiments.

![Figure 5.17 $^1\text{H},^{15}\text{N}$ HSQC for PfCuP-D3.](image)
5.3.4 Heteronuclear NOE relaxation of \textit{PfCuP-D3}

Nuclear Overhauser effect (NOE) originates from the dipole–dipole relaxation interactions between two spin-1/2 nuclei. This implies that the NOE is dependent on motions and the distances between the nuclei. NOEs can thus be used for the assessment of intramolecular motions, measurements of distances and structure calculation. Specifically, heteronuclear relaxation can thus be used to provide a detailed information about the internal dynamics of the protein.\textsuperscript{8} The \textsuperscript{1}H-\textsuperscript{15}N NOEs of \textit{PfCuP-MBD3} (Figure 5.18) reveals that about 20\% of the protein is dynamic and Figure 5.19 shows the dynamics of each residue in \textit{PfCuP-MBD3}. Heteronuclear NOE values were calculated as the ratio of peak heights in spectra recorded with and without the application of a saturation pulse to the nitrogen atoms.

![Figure 5.18 1H-15N Heteronuclear NOE relaxation revealing about 20\% of the PfCuP-D3 residues to be dynamic.](image1)

![Figure 5.19 1H-15N Heteronuclear NOE relaxation showing some detailed dynamics of PfCuP-MBD3. Negative 1H-15N NOE values, reveals regions that are extensively dynamic on the nanosecond time scale, which is typical of random-coil regions.](image2)
5.3.5 Triple resonance experiments and resonance assignments

Backbone resonance assignments were obtained by primarily using the CBCANH and CBCA(CO)NNH spectra, but were supported by information derived from the HNCA, HN(CO)CA, HNCO, and HN(CA)CO spectra. Resonances were assigned to the backbone atoms in 74 of the 89 amino acid residues in the PfCuP-D3 construct (Figure 5.20).

Figure 5.20 Resonance assignments for PfCuP-D3.
The general approach used to obtain resonance assignments was described in section 4.7.2. Table 5.1 list the chemical shifts of the amide atoms of each amino acid that was assigned using the triple resonance experiments collected on PfCuP-D3. Resonances that could not be assigned with 100% certainty, due to lack of clear connectivity, were left blank. The protein started to degrade during the data collection, especially at the C-terminus, and resulted in the inability to assign several amino acid residues.
Table 5.1 $^{1}$H-$^{15}$N chemical shifts for PfCuP-D3

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Table 5.1 $^1$H-15N chemical shifts for PfCuP-D3—Continued

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5.3.6 Secondary structure prediction based on chemical shifts

The secondary structure of PfCuP-D3 was predicted using PINE-SPARKY using the assigned chemical shifts shown on Table 5.35. The prediction indicates that the first 75 residues of PfCuP-D3 has a ferredoxin fold. This corresponds to the amino acids residues 480 -550 of the native protein (Figure 5.36).

![Secondary structure prediction](image)

Figure 5.21 Secondary structure prediction based on the chemical shift assignments. The prediction reveals that PfCuP-D3 has ferredoxin fold, $\beta\alpha\beta\alpha\beta$. 151
5.4 Biophysical characterization of *PfCuP-MBD2-3*

5.4.1 Circular dichroism measurements of *PfCuP-MBD2-3*

10μM of *PfCuP-MBD2-3* was used for the CD wavelength scan. Results shows that the *PfCuP-MBD2-3* has maxima at 198nm and three minima at 212 nm, 218 nm and 222 nm (Figure 5.22).

5.4.2 Chemical denaturation of *PfCuP-MBD2-3* using GuHCl

It was determined that *PfCuP-MBD2-3* unfolds in a three state model (Figure 5.23). This is because Domains 2 and 3 are independently unfolded.

Figure 5.22 CD spectra for *PfCuP-MBD2-3*. The alpha helical content was determined to be 12%

Figure 5.23 *PfCuP-MBD2-3* unfolds in three transition states. The protein forms a stable intermediate (I) as it transitions from its native (N) to denatured state (D). A plot of CD signal against the denaturant concentration give a double sigmoidal curve showing the transition of the protein from N→I and I→D. These transitions will have different ΔG and m values. These were obtained from the curve fitting of the plot to equation 5.19
Eq. 5.19

\[
S_{\text{obs}} = \frac{S_N + S_1 \exp\left\{ -\left( \frac{\Delta G_{N \rightarrow I} - m_{N \rightarrow I} [\text{GuHCl}]}{RT} \right) \right\} + S_D \exp\left\{ -\left( \frac{\Delta G_{I \rightarrow D} - m_{I \rightarrow D} [\text{GuHCl}]}{RT} \right) \right\} \exp\left\{ -\left( \frac{\Delta G_{I \rightarrow D} - m_{I \rightarrow D} [\text{GuHCl}]}{RT} \right) \right\}}{1 + \exp\left\{ -\left( \frac{\Delta G_{N \rightarrow I} - m_{N \rightarrow I} [\text{GuHCl}]}{RT} \right) \right\} + \exp\left\{ -\left( \frac{\Delta G_{N \rightarrow I} - m_{N \rightarrow I} [\text{GuHCl}]}{RT} \right) \right\} \exp\left\{ -\left( \frac{\Delta G_{I \rightarrow D} - m_{I \rightarrow D} [\text{GuHCl}]}{RT} \right) \right\}}
\]
The free energies of unfolding for PfCuP-MBD2-3 are summarized in Table 5.2

**Table 5.2: Free energies and m values of denaturation of PfCuP-MBD2-3**

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<tr>
<th>Transition state</th>
<th>ΔG (kJmol⁻¹)</th>
<th>m (kJmol⁻¹)</th>
<th>[GuHCl]</th>
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<td>N→I</td>
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<td>6.3±0.6</td>
<td>1.3±0.3</td>
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<td>I→D</td>
<td>21.4±3.1</td>
<td>7.8±0.6</td>
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The transition at 1.3 M GuHCl corresponds to the unfolding of domain two (Section 5.1.7) while the transition at 4.9 M GuHCl corresponds to the unfolding of domain three (Section 5.3.2).

### 5.4.3 Thermal unfolding of PfCuP-MBD2-3

Thermal unfolding for PfCuP-MBD2-3 was performed on a 10 µM sample. Temperature was increased gradually from 25°C to 95°C at a rate of 1°C/min and the CD signal was recorded at 222 nm. Figure 5.24 shows a sigmoidal curve for the thermal denaturation of PfCuP-MBD2-3. The temperature midpoint of the protein was found to be 47.9 ±0.7°C.

![Figure 5.24](image.png)

*Figure 5.24 Thermal unfolding for PfCuP-MBD2-3.*

The thermodynamic properties, ΔH and ΔS were obtained from the Van’t Hoff relation, equation 5.14. Keq was calculated from equation 5.12 and 5.13. A plot of ln(Keq) against T⁻¹ gives a linear curve where enthalpy ΔH can be obtained from the slope, and the change in entropy ΔS will be obtained from the y-intercept (Figure 5.25).
5.4.4 Copper metal binding affinity of *PfCuP-MBD2-3*

*PfCuP-MBD2-3* has one metal binding motif (MXCXXC) at domain 2. Purified protein was reduced with excess DTT and titrated against the Cu[^I][BCS]_2[^3-] complex. The binding of copper to the protein was followed by measuring the decrease of absorbance at 483 nm. The experiment was done under anaerobic conditions, as described in section 4.8. Two sets of titrations were performed at 400 µM Cu[^I][BCS]_2[^3-] and 500 µM Cu[^I][BCS]_2[^3-]. Figure 5.26 shows a plot of [Cu[^I][BCS]_2[^3-]] versus *PfCuP-MBD2-3*: Cu. The data was then fit to a curve according to equation 5.18 (Figure 5.27 and 5.28).

![Graph showing the decrease of absorbance](image1)

**Figure 5.25** Determination of free energy of thermal unfolding for *PfCuP-MBD2-3*. Using equation 5.15, free energy of thermal denaturation of *PfCuP-MBD2-3* at 298 K was found to be 17.9 ± 0.4 kJ/mol.

![Graph showing titration](image2)

**Figure 5.26** Titration of *PfCuP-MBD2-3* against 500 µM BCS (red spheres) and 400 µM BCS (black spheres).
Figure 5.27 Curve fitting of the titration of PfCuP-MBD2-3 against 500 μM [Cu(BCS)₂]³⁻. K_D was determined to be 2.4 * 10⁻¹⁸ M.

Figure 5.28 Curve fitting of the titration of PfCuP-MBD2-3 against 400 μM [Cu(BCS)₂]³⁻. K_D was determined to be 1.3 * 10⁻¹⁸ M.
5.5 Summary

This chapter discussed the biophysical characterization of the N-terminal domains of \( PfCuP \)-ATPase using circular dichroism, metal affinity assays and solution NMR spectroscopy. The alpha helical content of each domain as calculated using circular dichroism, ranges between 12-27% which is within the range typical of globular proteins, which have an average of 30% alpha helical content.\(^9\) The stability of the protein constructs was assessed using chemical denaturation with guanidine hydrochloride and thermal denaturation, by gradually heating the protein from 25°C – 95°C. Domain 1 of \( PfCuP \)-ATPase retained 50% of its structure at 2.8 M GuHCl, Domain 2 is the least stable since it begins to unfold with only 0.35 M GuHCl, and retains 50% of its structure at 1.35 M GuHCl. Domain 3 is the most stable of the three domains since it retains 50% of its structure at 4.6 M GuHCl. Domains 1 through 3 unfold in a two phase manner as concentration of the concentration of denaturant is gradually increased. When domains 2 and 3 are joined together as one protein construct, the protein displays a double sigmoidal unfolding curve. The first midpoint is consistent to the unfolding of domain 2 where 50% of the protein retains its structure at 3 M GuHCl, and the second midpoint at 4.9 M GuHCl, that corresponds with the unfolding of domain 3. Only Domain 1 and Domain 2-3 constructs were thermally denatured. Domains 2 and 3 constructs precipitated as they were heated. This may be due to structural rearrangement of the domains that might have occurred during heating that may have resulted in the aggregation of the proteins. Solution NMR and the circular dichroism data corroborates the prediction that \( PfCuP \)-ATPase has three independent N-terminal domains.
Metal binding affinity assays shows that constructs for domain 2 and a construct of Domain 2 and 3 joined together are able to bind copper with a high affinity. Domain 2 has K\text{D} of 1.35 * 10^{-18} M while the affinity for copper for Domain 2-3 construct was found to be 1.85 * 10^{-18} M. The copper affinity for PfCuP-MBD2 and PfCuP-MBD2-3 compares well with copper affinity for Atox1 and Wilson disease domain 5-6.\(^4\)

Triple resonance experiments were acquired on Domain 3 and the resonances assigned, as detailed in section 4.3.5. Domain 3 was cloned and had a total of eighty-nine amino acids residues, spanning residue 476 to 564 in the full length PfCuP-ATPase. As seen from Table 5.5, resonances that were assigned are between residue numbers 479 and 549. It was determined that the protein began to degrade and did not remain stable at the end of the data collection week. This confirmed by a lack of connectivity during the assignment of resonances corresponding to C-terminal residues. Future experiments should be repeated with protease inhibitors added. However, since the majority of the peaks were assigned, it is possible to obtain a CS Rosetta predicted model of the structure. Using PINE SPARKY software, secondary structure prediction of PfCuP-D3 based on the chemical shift assignments reveals that PfCuP-D3 has ferredoxin fold, \(\beta\alpha\beta\alpha\beta\).
5.6 REFERENCES


CHAPTER 6
COPPER BINDING AFFINITIES OF DICTYOSTELIUM DISCOIDEUM COPPER CHAPERONE ATOX1 AND METAL BINDING DOMAIN ONE OF COPPER P-TYPE ATPASE 1

6.1 Introduction

*Dictyostelium discoideum* is a slime mold, free-living amoeba that inhabits the upper layer of forest soil that is rich in organic material. It feeds mainly on bacteria by phagocytosis and thus could be a natural source of pathogenic bacteria. During starvation, amoeba solitary cells aggregate through chemotaxis to form multicellular organisms.\(^1\)\(^2\) The multicellular organisms, called slugs, migrates towards warmth and light in search of a suitable environment for fruiting body formation.\(^1\) These cells differentiate into pre-stalk and pre-spores that migrate and grow into a fruiting body made of slender stalks and fully differentiated spores on top. Because of their easy handling and amenability to genetic manipulation, *Dictyostelium discoideum* has been a valuable model organism for studies such as motility, chemotaxis, morphogenesis, and host resistance to infection and the hosts’ interaction with many clinically relevant bacterial pathogens.\(^3\)\(^4\)\(^5\)

6.2 Metal ion homeostasis in *Dictyostelium discoideum*

Various divalent transitional metal ions have been involved in host pathogen interactions, such as, manganese, copper and zinc.\(^6\) Iron and manganese are depleted from the mature phagosome, while copper and zinc are thought to be pumped into the phagosome to intoxicate the bacteria. This is supported by the finding that increased concentrations of copper and zinc are found in *mycobacterial* infections.\(^7\) The development of *Dictyostelium discoideum* is sensitive to the presence of heavy metals in the soil. Higher
concentrations of mercury (50 mg/kg of soil) inhibit protozoan growth, more so than high concentrations of manganese, iron and copper.\(^8,9\)

6.3 Copper ion metabolism in Dictyostelium discoideum

*Dictyostelium discoideum* is a social amoeba has three genes for copper ATPases: ATP1 (gene ID: 8618898) which encodes a 1280 amino acid residue protein, ATP7A, (GenBank: EAL65411.1) which encodes a protein of 985 amino acid residues and ATP3, GenID: 8617046 which encodes a protein with 1386 amino acid residues. The *Dictyostelium* genome encodes also encodes a putative copper transporter, p80, that is localized in the plasma membrane and in the phagosome. The p80 copper transporter is a Ctr1 homologue. Burlando et al. discovered that *Dictyostelium discoideum* has unusual copper resistance. The resistance was associated with the presence of a copper ATPase homologue of MNK protein, which uses copper ions to kill bacteria.\(^10\) Other possible copper proteins (DDB_G0288281gene ) present in the protozoan are metallothioneins, which are cysteine-rich proteins that binds heavy metals through their thiol groups. The presence of metallothioneins is yet to be determined. Burlando et al. used a cytochemical technique and Western blotting to demonstrate the occurrence of Cu-ATPase activity. The 105 kDa apparent molecular weight of the ATPase determined by Western blot, agrees well with the Cu-ATPases with a molecular weight of 100 kDa to 150 kDa .\(^10\)

6.4 Involvement of copper and zinc metal ions in the phagosomal killing of bacteria

The copper ion is used by many metalloenzymes, but an excess is detrimental to cells. Prokaryotes must have mechanisms that maintain copper homeostasis, and allow the accumulation of ions in the intracellular organelles and their eventual utilization in a particular biological activity. Macrophages utilize Zn(II) and Cu(I) to destroy Fe-S
clusters that are essential for the survival of bacteria. The entry of copper ions in the amoeba is facilitated by a copper transporter protein p80, a membrane bound protein which is homologous to human and yeast copper transporter 1. By analogy to yeast and humans, inside the protozoan cell, a copper chaperone Atox1 receives the Cu(I) ion and transports it to ATP7A, a TM protein that is localized in the trans Golgi network. ATP7A eventually transfers the copper ions to the phagosome where it is involved in killing and digesting of bacteria. As a defense mechanism, the bacteria also express copper ATPase (CopA) for the efflux of the copper ions, and a Zinc ATPase (ZntA) for the efflux of the zinc ions (Figure 6.0).

![Figure 6.0 Metal ions and their involvement in phagosomal bacterial killing.](image)

11, 12, 13, 14
*Dictyostelium discoideum* expresses three copper ATPases: ATP7A, ATP1, and ATP3. ATP7A is localized in the membrane of TGN while ATP1 and ATP3 may also be localized in the TGN, however their exact localization has not been elucidated. ATP7A, ATP1 and ATP3 bind copper in the form of Cu(I). The removal of iron and manganese ion from the phagosome is facilitated by the natural resistance-associated macrophage protein 1 (NRMP1). *E.coli* expresses ZntA and CopA used for the efflux of zinc and copper ions respectively. CusCBA is required for the removal of the periplasmic copper. There is little information available on the ATP7A, ATP1 and ATP3 copper ATPases of the *Dictyostelium discoideum*.

### 6.5 N-terminal domain of *Dictyostelium discoideum* ATP1

ATP1 has 1280 amino acid residues. The N-terminal portion of the protein has two copper binding motifs (MKCXXXC and MTCXXC). The protein sequence was analyzed using Jpred 4 (a protein secondary structure prediction server) to predict the structured regions of the protein. The sequences of the structured portions of the protein were then modelled using Phyre2 (an online server for predicting the 3-dimensional structure of a protein) and SWISS-MODEL (an online server for protein structure homology-modelling). Of all the models of homologues from Phyre2, a model of a template with the highest degree of confidence (100%) and percent identity were selected and processed further using PyMOL™. The resulting prediction of the metal binding domains of ATP1 are shown in Figures 6.1 and 6.2. In all the models Wilson disease protein metal binding domains (PDB: 2EW9 and 2ROP) were used as the template. Modeling of this ATP1 suggested the protein has two metal binding domains. These two
domains are predicted to have 69 residues (residues 201-270 for Domain 1) and (residues 356-425) for Domain 2 respectively. The domains are predicted to have a ferredoxin fold similar to the Menkes and Wilson disease proteins metal binding domains. Domains 1 and 2 were modeled using a template of solution structures of the Wilson disease protein domain 5 and 6 (PDB: 2EW9), and domain 3 and 4 (PDB: 2ROP).

![Image](image1.png)

Figure 6.1 N-terminal metal binding domain 1 of ATP1 with MKCXXXC copper binding motif. The cysteine residues, shown in sticks are located in the loop connecting $\beta_1$ and $\alpha_1$.

![Image](image2.png)

Figure 6.2 N-terminal domain 2 of ATP1 with MTCXXC copper binding motif. The two cysteine residues are shown in sticks are located at the end of the loop of $\beta_1$ and at the beginning of $\alpha_1$ helix.

### 6.6 Dictyostelium discoideum copper chaperone Atox1 (DdAtox1)

*Dictyostelium discoideum* has a copper chaperone (*DdAtox1*) for delivery of Cu(I) to ATP7A. The delivery of Cu(I) to ATP1 and ATP3 has been documented in literature. *DdAtox1* has 67 amino acid residues. It is predicted to have a ferredoxin fold similar to human copper chaperone (HAH1).
6.7 Expression and purification of DdAtox1 and the metal binding domains 1 and 2 of ATP1

Plasmids containing the genes encoding DdAtox1 and the N-terminal domains were purchased from GenScript Inc. The genes were synthesized in order to utilize codon optimization and overcome anticipated protein expression challenges in E. coli. The synthesized genes were cloned into the pET-28(b) expression plasmid, which has a Histidine tag at the C-terminus. The TEV protease site was introduced at the end of the protein such that the affinity tag will be cleaved off with the protease during the purification steps. BL21(DE3) competent cells were transformed with 60 ng of plasmid DNA. The colonies were grown overnight at 37°C for 16 hours. One colony was then used to inoculate a starter culture of 5 mL LB media with kanamycin. The starter culture was grown for 6 hours then scaled up to 500 mL LB growth media in a 2 L flask. The protein expression was induced by addition of IPTG when O.D at 600 nm was 0.6. The protein was expressed for 5 hours at room temperature. The cells were harvested by centrifugation and the cells were resuspended in a lysis buffer A containing 30 mM sodium phosphate buffer pH 7.5, 150 mM sodium chloride, 0.5 mM EDTA. The cells were ruptured by sonication and then centrifuged. The supernatant containing the soluble proteins was loaded into a HisTrap HP column (GE Healthcare Life Sciences) pre-equilibrated with binding buffer: 30 mM sodium phosphate buffer pH 7.5, 150 mM sodium chloride, and 20 mM imidazole. The column was washed with five column volumes of binding buffer. The protein was then eluted by gradient using elution buffer: 30 mM sodium phosphate buffer pH 7.5, 150 mM sodium chloride, and 300 mM imidazole. Fractions containing DdAtox1 were combined together, concentrated by
Amicon filtration device fitted with 3.0 kDa NMWL (nominal molecular weight limit) membrane. The protein was then buffer exchanged into 50 mM Tris-HCl, pH 8.0. Protein concentration was determined by BCA assay and the TEV protease added at a mass ratio of 80:1, (protein/protease). The protein was digested overnight at room temperature and the protein was passed through HisTrap column to remove the His<sub>6</sub> tag. Protein without His<sub>6</sub> tag was collected as a flow through, then buffer exchanged into 30 mM sodium phosphate, 150 mM sodium chloride, 1 mM DTT, pH 7.5. Protein yield was 11.6 mg for a 1L growth media.

For <sup>1</sup>H-<sup>15</sup>N HSQC NMR sample preparation, the protein was expressed and purified in the same manner as described in section 4.4.1, except the protein was expressed in *E. coli* in M9 minimal media, which contained isotopically labeled ((<sup>15</sup>NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) as the only nitrogen and carbon source respectively. The purity of the proteins was assessed using SDS-PAGE (Figure 6.3).

![Figure 6.3](image)

Figure 6.3 (a) Purification of *Dd*Atox1. L; protein marker, 1; cell lysate, 2; His trap fraction, 3&4; pure *Dd*Atox1 without the affinity tag. (b) Purification of *Dd*ATP1-MBD1. L; protein marker, 1; cell lysate, 2; His trap fraction, 3 &4; pure protein after removal of affinity tag. (c) Purification of *Dd*ATP1-MBD2. L; protein marker, 1; His trap fraction, 2; pure protein without the affinity tag.
6.8 Heteronuclear Single Quantum Coherence ($^1$H-$^{15}$N HSQC) for *DdATP1-MBD1*, *DdATP1-MBD2* and *DdAtox1*

Purified $^{15}$N labelled proteins were buffer exchanged into 30 mM sodium phosphate, 150 NaCl mM, and 1 mM DTT, pH 7.5 using PD10 desalting columns. Protein were concentrated to 0.6 mM for NMR spectroscopy studies. All buffers used were made fresh and degassed before use. Dr. Blair Szymczyna collected the NMR spectra on a Bruker AVANCE II 800 MHz NMR at the University of Notre Dame by Dr. Blair Szymczyna. The data was processed using NMRPipe. The $^1$H,$^{15}$N HSQC spectra for *DdAtox1*, *DdATP1-MBD1* and *DdATP1-MBD2* reveals that the resonances are well dispersed, which a characteristic of a structured protein (Figures 6.4, a, b and c).

Figure 6.4 $^1$H-$^{15}$N HSQC spectra for (a) *DdAtox1*, (b) *DdATP1-MBD1*, and (c) *DdATP1-MBD2*
6.9 Copper metal binding affinity of DdATP1-MBD1 and DdAtox1

Purified proteins were titrated against two different concentrations of competing ligand bathocuproinedisulfonate (BCS), which forms a 2:1 complex with Cu\textsuperscript{1}. All copper binding experiments were performed under anaerobic conditions in a glovebox as described in Section 4.7.4 Figures 6.5 - 6.10 shows the corresponding titration curves of the [Cu\textsuperscript{1}(BCS)\textsubscript{2}]\textsuperscript{3+} against protein copper ration and the curve fitting to equation 5.18 in order to determine the affinity constants...
Figure 6.5 Titration of 
*Dd*Atox1 against
*[^1](BCS)\textsuperscript{2}[^3-]* (500 µM, 
Black squires and 400 
µM, red spheres)

Figure 6.6 Curve fitting of the 
titration of *Dd*Atox1 to 400 
µM *[^1](BCS)\textsuperscript{2}[^3-]*. \( K_D \) was 
found to be \( 1.4 \times 10^{-18} \) M.

Figure 6.7 Curve fitting of the 
titration of *Dd*Atox1 to 500 
µM *[^1](BCS)\textsuperscript{2}[^3-]*. \( K_D \) was 
found to be \( 2.01 \times 10^{-18} \) M.
Figure 6.8 Titration of copper DdATP1-MBD1 with [Cu\((\text{BCS})_2\)]\(^3\)- (500 µM, blue spheres and 400 µM, red spheres).

Figure 6.9 Curve fitting of the titration of DdATP11 to 300 µM [Cu\((\text{BCS})_2\)]\(^3\). \(K_D\) was found to be \(2.1 \times 10^{-18}\) M.

Figure 6.10 Curve fitting of the titration of DdATP1 to 400 µM [Cu\((\text{BCS})_2\)]\(^3\). \(K_D\) was found to be \(2.4 \times 10^{-18}\) M.
6.10 Summary and future work

In this work, it has been shown that the N-terminal domain of copper ATPase 1 of *Dictyostelium discoideum* has two metal binding domains. This is evidenced by the Heteronuclear Single Quantum Coherence (\(^1\)H-\(^{15}\)N HSQC) spectra (Figure 6.8) that suggests the domains have a tertiary structure. The binding affinity values calculated from the curve fitting of the titrations against 400 and 500 µM \([\text{Cu}^1(\text{BCS})_2]^{3-}\) were averaged and adopted as the affinity constants of the protein. The affinity constant for metal binding domain one of *Dd*ATP1 was found to be \(2.3 \times 10^{-18}\) M while that of the copper chaperone *Dd*Atox1 was found to be \(1.7 \times 10^{-18}\) M. The metal affinity for *Dd*Atox1 and *Dd*ATP1 are similar to those reported in literature for Wilson disease domains 5-6 and Atox1.\(^{15}\) These result taken together shows that Domain 1 of *Dd*ATP1 has a higher affinity for copper compared to the metal chaperone *Dd*Atox1. This is particularly important for the chaperone to be able to donate the copper ion to the efflux protein. The binding affinity of the second domain has not yet been determined. Future experiments could be designed compare the affinity for metal binding domain two which has the conserved MXCXXXC motif for metal binding in contrast to Domain 1 that has the MXCXXXXC motif, and whether Domain 1 can transfer the copper ion to Domain 2. There is no structure of a metal binding domain of P-type Copper ATPase with a MXCXXXXC motif. It would be of interest to determine the structure of this domain and and determine if it adopts a ferredoxin fold, which is a characteristic of the N-terminal domains of copper transport proteins.
6.11 REFERENCES


CHAPTER 7
DISSERTATION SUMMARY AND FUTURE WORK

7.1 Dissertation summary

Copper transport proteins are required for the maintenance of the copper levels in living systems. Understanding structural organization of these copper ATPases sheds more light on their mode of action. In this work, four copper transport proteins have been studied. Wilson disease protein, ATP7B, which is involved in the copper metabolism in humans was cloned and expressed in E. coli. Six expression vectors were prepared and different strains of E. coli competent cells tested for the expression of the ATP7B gene. The full length ATP7B gene lacking the first 57 amino acid residues was amplified by PCR and the insert DNA of 4235 bp was cloned into β-pET28a(+) plasmid, a derivative of pET28a(+) vector encoding two fusion domains, α and β, that enhances the expression of membrane proteins on E.coli. Small scale protein expression was achieved with C41(DE3) and C43(DE3) strains of E. coli cells at 18°C. Attempts to scale up the expression was unsuccessful. It was observed that the large scale expression of the protein resulted to cell death and the project was no longer feasible.

In the second project, metal binding domain four of Wilson disease protein was fused to a copper chaperone, HAH1 to form a 20.7 kDa chimera of two domains separated by 57 residue linker. The protein was found to be very stable and relatively resistant to chemical and thermal denaturation. Using Guanidine chloride as a denaturant, the protein was shown to unfold into three phase transition states: native intermediate and denatured state respectively, as the concentration of the denaturant was increased. The mid-point for the first transition occurs at 1.37 M GuHCl while the second mid-point transition occurs at 5.6 M GuHCl. This corresponds to the unfolding
of HAH1 and domain four of Wilson disease protein respectively. Gibbs free energy of chemical unfolding was revealed to be 6.1 kJ/mol in the first transition and 44.8 kJ/mol in the second transition. The higher energy of unfolding in the second transition is due to the high stability of domain four which unfold in the second transition. Thermal unfolding of the chimera was monitored at 222 nm as the protein was heated gradually from 25°C to 98°C. It was revealed that the thermal mid-point of unfolding of the chimera is 78°C.

Following stability studied of the chimera, crystallization trials were performed at room temperature using Hampton’s crystallization screens by hanging drop diffusion method. Crystallization was initiated using horse hair as a nucleating agent. Crystals that grew on horse hair were harvested and used for seeding to grow secondary crystals. The crystals were exposed to an X-ray beam at the advanced photon source at Argonne national laboratory. Poor diffraction of the crystals made it impossible to obtain diffraction data for structural analysis.

The third project involved the determination of the N-terminal domains of *Plasmodium falciparum* copper ATPase (*PfCuP*-ATPase). Copper metabolism in *Plasmodium falciparum* is regulated by a *PfCuP*-ATPase, a copper efflux protein that reduces copper toxicity. *PfCuP*-ATPase is the largest copper transporter of the P~IB~ P-type ATPase family made up of 2563 amino acid residues with a molecular weight of 298.8 kDa. The number of metal-binding domains in copper ATPases varies among organisms. In *PfCuP*-ATPase, the number of metal-binding domains and how the copper ATPase functions is only partially understood. Using bioinformatics tools, circular dichroism and solution NMR, I have identified three N-terminal domains of *PfCuP*-ATPase found within the first 564 amino acids. Surprisingly, only one domain contains the copper binding motif MXCXXC. There is a long linker of 320 amino acids between the first and the second domains, and the second domain is directly adjacent to domain three.
I pursued biophysical studies to better understand the structural organization of these domains. Each domain was separately cloned using recombinant technology and expressed in E. coli. Domain three is the most soluble and relatively resistant to chemical denaturation with guanidine hydrochloride. In contrast, domain two is less stable. It retains only 50% of its helical structure at 1.35 M GuHCl. Strikingly, domain one is resistant to thermal denaturation and it has a melting midpoint of 78°C. Domains two and three, when heated, precipitate out of solution. However, when they are expressed together as a two-domain construct (PfCuP-MBD2-3), thermal denaturation shows a sigmoidal curve with a melting midpoint of 47.9°C. The chemical denaturation of PfCuP-MBD2-3 produced a double sigmoidal curve and the second sigmoid correlates to the denaturation of domain three. Copper binding studies were performed under anaerobic conditions with Cu(CH₃CN)₄⁺ as the copper source, and I obtained a K_D of 1.35 * 10⁻¹⁸ M. The copper ATPase of P. falciparum diverges from that of the kingdom Animalia. The presence of a two domain construct, like that of human Wilson protein metal-binding domains five and six, yet lacking a metal-binding motif in domain three is intriguing. This finding highlights the structural versatility and stability of the dual ferredoxin fold.

¹⁵N-labelled samples were prepared and ¹H-¹⁵N HSQC NMR experiments were performed for four different constructs. Subsequently, an ¹³C,¹⁵N sample of domain three was prepared and triple resonance NMR experiments were performed. NMR resonance assignments and subsequent Rosetta modelling shows domain three to have a ferredoxin fold that is similar to the metal binding domains of human Wilson disease protein.

The fourth project involved the determination of the copper binding affinities of N-terminal domains of Dictyostelium discoideum copper ATPase (DdATP1) and a copper chaperone for Dictyostelium discoideum copper ATPase (DdAtox1). In this project I was able to identify two N-
terminal metal binding domains of DdATP1. Domain 1 has unusual MXCXXXC copper binding motif that is not common in copper transport proteins. Domain 1 and 2 of DdATP1, and DdATox1 were cloned into pET24(d+) and expressed in E. coli. Proteins were purified using metal ion affinity chromatography. The ¹H-¹⁵N HSQC spectra revealed that the proteins are structured domains. Copper titrations experiments were done under anaerobic conditions in a glovebox. The copper binding affinity for Domain 1 of DdATP1 was found to be 2.3 * 10⁻¹⁸ M, while that of DdATox1 was 1.7 * 10⁻¹⁸ M. Table 7.0 summarizes the biophysical properties of each protein construct studied.

Table 7.0

<table>
<thead>
<tr>
<th>Protein construct</th>
<th>T_m(°C) Melting temperature</th>
<th>C_m(M) [GuHCl] at midpoint</th>
<th>ΔG_H₂O (kJ/mol) Gibbs free energy of chemical unfolding</th>
<th>ΔG_H₂O (kJ/mol) Gibbs free energy of thermal unfolding</th>
<th>K_D Dissociation constant</th>
</tr>
</thead>
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<tr>
<td>PfCuP-D1</td>
<td>68.1±3.6</td>
<td>2.8± 0.2</td>
<td>9.0± 0.8</td>
<td>8.8±1.3</td>
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<tr>
<td>PfCuP-D2</td>
<td>Protein aggregates</td>
<td>1.4±0.1</td>
<td>6.1±1.1</td>
<td></td>
<td>1.35 x 10⁻¹⁸M</td>
</tr>
<tr>
<td>PfCuP-D3</td>
<td>Protein aggregates</td>
<td>4.6</td>
<td>15.9±1.2</td>
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<td></td>
</tr>
<tr>
<td>PfCuP-D2-3</td>
<td>47.9±0.7</td>
<td>(1st) 1.3±0.3</td>
<td>(1st) 11.4±1.3</td>
<td></td>
<td>1.85 x 10⁻¹⁸M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2nd) 4.9±0.4</td>
<td>(2nd) 21.4±3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DdATP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.3 x 10⁻¹⁸M</td>
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<tr>
<td>DdATox1</td>
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<td></td>
<td></td>
<td></td>
<td>1.7 x 10⁻¹⁸M</td>
</tr>
<tr>
<td>WLN5-6 ¹</td>
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<td>Atox1 ¹</td>
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<td></td>
<td></td>
<td></td>
<td>4.4 x 10⁻¹⁸M</td>
</tr>
</tbody>
</table>
7.2 Research significance

In this work, three N-terminal domains of PfCuP-ATPase, and two N-terminal metal binding domains of DdATP1 have been identified. These domains have not been previously mentioned in literature and it is the first time they have been characterized. The results described in this work adds to the information available in literature and contributes to the scientific community towards understanding the structure and function of these enzymes. The existence of two cytosolic domains in PfCuP-ATPase lacking the metal binding binding motif is intriguing. This opens a new area of study to investigate the role of these domains in the overall function of the ATPase.

7.3 Future work

The knowledge gained in this work regarding the existence three N-terminal domains in PfCuP-ATPase could be pursued further in order to solve their solution or crystal structures and determine their function if they bind other metals, and their function in association with the rest of the protein. It will be interesting to determine their significance in the ATPase since this domain is absent in other copper ATPases. Other domains within the protein have also been identified as detailed in the sequence shown in figure 7.0.

Figure 7.0 PfCuP-ATPase sequence

```
MAKLSLTINNVDDDLKRKIKKAKKFEGVEDLKHKNKKTISYNPKVVDYSYGVIERIKHLDINGE
ILKDDNHESSKNNSFTFSMYKEGVEENVVKKANIFDDNENNQNTNNYIDNNSNNCNYGINN
NRDNYDKLDCNGNNADNNFDQIYDMNMNVMNYTYPNGEDLYEQLDNAYDDKNYKDNYKYKNTN
HQQCEQNYKNEAEIHQQEQQNYKQYKAEANHQQCEQNYKNEANHQQKSEQNYKSYGDNYQNCNVS
EKNENNSNENSNKGDLYDINYYPNQVKEVSESEGVDENKSSKFSLNLNFNIIKSDKKK
KMKEIKEKKDYLNEYNNLDRKILLSDMYKNVENKNYNNSNDDNYSSNYYDYNEEKLKKN
FNFRDFYESYRDKQNIYICEKIIYMTCDNGKKKIIINFTKLGKLLIEGNSFATDNKIKLKDIDS
SDIHCNNNVKMVFNTMNEIKESGFNNLDLDYKDDNKNCRNLSEITLYIYRDDDIEKKSYYLKL
DIKGISNVEYDLKKEIYFLYFELPGIRYILEIKKKKNVDAYYDEDKEYFRSSQNNENNN
TWKLIESLICIIIISIIIIVLNYQOMNGSMNFYSYTKKLFDNNSKIDTNDKPHSIYHIGN
GTPVDFQSTLDIYFENVTKGLTNVHKOQSRDEKEGTFFINSNFKIKKDPSINRKKWVDDD
IKKQILNGKKNKSIYNTINMLNQIDAGYNNQEGHDKYPLDNNVNSKYIETCNSENRR
KNYLNEKDDDDNKKENINNNNDNNDKNDNDFNNDYNKDDYDEHYDPIGDFRKSIDE
```
Figure 7.0 PfCuP-ATPase sequence Continued

GLSLGCTRTNVYVMDASADACIVDNPSNVFSGFLFTFLMFCOUNTIDTYPFYLKYYCNKQPYIEEKLTVKSMERIYSCD
CNVHKTVQYLYYSNNSKKNESNIIIFMCGIEGIVGVFFTLDVNIKPEVFELINFIIKREKKVYVCYGDNYMHALYISKI"

Figure 7.0 Protein sequence for PfCuP-ATPase. Colored portions of the protein represent identified domains.
The two identified N-terminal metal binding domains of *DdATP1*, there is need to determine if there exists inter-domain interaction, and whether Domain 1 can transfer copper to Domain 2. This would shed more light as to why their metal binding motifs differ, and compare their metal binding affinities. Furthermore, in this work, the copper binding affinity of the copper chaperone *DdAtox1* was revealed to be higher than that of Domain 1. This may imply that the chaperone may be able to donate the copper ion to this domain. Whether it can donate the metal ion to either of the domains, has not been determined.
7.4 REFERENCES

APPENDIX

Project approval certification
Institutional Biosafety Committee

2016
Project Approval Certification

For Institutional Biosafety Committee Use Only

Project Title: Characterization of Metalloproteins

Principal Investigator: David Huffman

IBC Project Number: 16DHD

Date Received by the Institutional Biosafety Committee: November 25, 2015

☑ Reviewed by the Institutional Biosafety Committee

☑ Approved
☐ Approval not required

Sylwia Rayboch
Chair of Institutional Biosafety Committee Signature

Date: 12/11/2015