FUNCTION AND CELLULAR TRANSPORT OF IRON CHEMISTRY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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*****

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In this research, we primarily focus on the structure and function of both the nucleotide binding domain, and the full length membrane-spanning transporter. First, the soluble nucleotide binding domain of Atm1 (Atm1-C), an ABC transporter in yeast mitochondria, that has previously been implicated in the maturation of cytosolic iron-sulfur cluster proteins, has been overexpressed in *E. coli*, purified, and characterized.

The full length version of Atm1 from *Saccharomyces cerevisiae* has been cloned, over-expressed, purified from a yeast expression system, and characterized. A fluorescent assay of liposome-loaded reconstituted Atm1p suggested that Atm1p only allowed small molecules and/or metal complexes to cross the channel. Both pH gradient and fluorescent assays also indicated that ADP-bound Atm1p existed in an open state that is different from the closed state for ATP-bound Atm1p.

The further discovery of an iron carrying peptide, hepcidin, provides the first step toward understanding iron trafficking in living cells. With eight, well-conserved cysteine residues in the sequence, hepcidin may not only be a signal peptide, but could potentially serve as an iron carrier. The iron binding properties have been determined by UV-vis spectroscopy, mass spectroscopy, and isothermal titration calorimetry (ITC). The iron binding affinity has been determined in the micromolar range. Studies by circular
dichroism (CD) reveal varying degrees of secondary structure within an apparent
dynamic tertiary fold. Taken together, hepcidin clearly binds iron, and the secondary
structure change induced by iron binding may be required for the full function of the
peptide in iron homeostasis and antimicrobial activity.

In Part II, a novel fluorescent assay has been developed for monitoring the
cleavage of a target RNA by cooper kanamycin *in vivo*. However, demonstration of the
efficacy of such reagents *in vitro* is only a first step. To demonstrate *in vivo* cleavage
chemistry we have designed a fluorescence assay based on use of the green fluorescent
protein (GFP). The decrease in fluorescent intensity indicated the designed target RNA
being destructed. The success in this assay provided a novel tool for screening a large
quantity of drug molecules for their targets at the same time.
Dedicated to my wife Shiau-Ling, daughter Anna, son Aaron, and my parents
I am deeply indebted for my advisor, Dr. James A Cowan, who has provided me the great support and encouragement during my PhD research at Ohio State University. Dr. Cowan is a great mentor with his patience and enthusiasm who has provided me a valuable learning experience during my PhD research and taught me the knowledge of science. I would like to thank him give me this great opportunity to pursue my research dream.

During my PhD research at Ohio State, I would like to thank my classmate, Manunya, for help with ITC and CD spectroscopy, Craig Henmann from Dr. Hille group for acquiring the EPR data, Kshama for running HiPIPs NMR samples, and Dr. Kari Green-Church for ESI-MS samples. I would also thank the past and present group members who have help me for my experiments.

I would like to thank Department of Chemistry for financial support. Finally I would like to thank my wife Shiah-Ling, daughter Anna, son Aaron, and my parents who are always there supporting my research during past years.
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PUBLICATIONS

Research Publications


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LIST OF ABBREVIATIONS

α  alpha
ABC  ATP-binding cassette
ATP  adenosine triphosphate
β  beta
BSA  bovine serum albumin
CD  circular dichroism
CTP  cytidine triphosphate
°C  degrees Celsius
δ  chemical shift in parts per million downfield from tetramethylsilane
DNA  deoxyribonucleic acid
DMSO  dimethylsulfoxide
DTT  1,4-dithiothreitol
E. coli  *Escherichia coli*
EDTA  ethylenediaminetetraacetic acid
EPR  electron paramagnetic resonance
ESI  electronspray ionization
FMN  flavin mononucleotide
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<td>FPLC</td>
<td>fast protein purification liquid chromatography</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
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<td>HiPIP</td>
<td>high potential iron protein</td>
</tr>
<tr>
<td>HisP</td>
<td>histidine permease</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl thiogalactoside</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<td>NBD</td>
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<td>Ni-NTA</td>
<td>nickel N-(5-amino-1-carboxypentyl)iminodiacetic acid</td>
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<td>NMR</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
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<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>phenylmethanesulfonyle fluoride</td>
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<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<td>UTP</td>
<td>uridine triphosphate</td>
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<td>----------------------------------------</td>
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<tr>
<td>UV-vis</td>
<td>ultraviolet-visible</td>
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<td>YFH</td>
<td>yeast frataxin homologue</td>
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<td>YPD</td>
<td>yeast extract peptone dextrose</td>
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CHAPTER 1

OVERVIEW

1.1 INTRODUCTION

Iron commonly exists in one of two oxidation states (+2 and +3). With the coordination to different ligands, there is a wide range of redox potential for iron complexes (1). This versatile ability for one-electron conversion between ferrous and ferric ions makes it a very important component for many redox enzymes or component of the electron transfer chain in the mitochondrion. When the reduction potential of iron complexes falls between the oxidants: +820 mV (the reduction potential of dioxygen at pH 7.0) and the reductants: -320 mV (the reduction potential of the pyridine nucleotides) in the biological system, a reversible redox reaction is feasible (2). Iron ranks fourth in abundance in the earth’s crust, but it is bio-unavailable under aerobic conditions, in which iron primarily exists in ferric state. The solubility product constant (Ksp) of ferric hydroxide (Fe(OH)_3) is around 10^{-38} (3). This means that at neutral pH, the solubility of ferric ions is only around 10^{-17} M. This may cause difficulty for us to acquire iron from our diet, since plants are the primary iron source for human. Fortunately, plants have developed two strategies to overcome this difficulty for iron uptake from the environment. The first strategy is to decrease the pH of...
the surrounding environment by use of a proton pump that increases the solubility of ferric ions by a thousand fold with one unit pH decrease in solution. Following the reduction of ferric ions to ferrous ions, this will increase the concentration of iron in the environment and provide enough iron for plant survival. The second strategy used by plants to acquire iron from their surrounds is to secret iron chelators. Phytosiderophores increases iron availability around the roots and facilitates iron uptake (Figure 1.1) (4). However, free iron can damage cellular proteins, DNA, and membranes via generated hydroxyl radicals by reaction with hydrogen peroxide (Scheme I) (4). Therefore, tight regulation of iron in the cell is necessary.

Scheme 1.1 Redox reaction of iron in vivo

Reduction of Fe$^{III}$ by superoxide anions

\[
\cdot\text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+}
\]

Fenton reaction

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH}
\]

Sum: Haber-Weiss reaction

\[
\cdot\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2
\]
Figure 1.1 Two strategies for iron uptake in plant roots. In strategy-I, ferric ion was first reduced by FRO2, a ferric ion chelate reductase, to ferrous ion, which is then taken up by IRT1, a ferrous ion transporter. Strategy-I plants include all higher plants except grasses. In strategy-II, the phytosiderophore-Fe$^{3+}$ complex is transported by YS1, a phytosiderophore-Fe$^{3+}$ transporter. Strategy-II plants include grasses, cereals, maize, and rice. (Taken from (4))
1.2 Mechanism of Iron Metabolism

The mechanism of iron homeostasis has been intensively studied for several decades. Nevertheless, the only well-established steps in iron homeostasis are iron uptake and storage. Iron trafficking and recycling inside the cell is still poorly understood. Since the yeast genomics have been intensively studied, and most yeast genes are conserved from prokaryotes to eukaryotes, the iron metabolism in yeast was used as a model system for comparison with mammalian iron metabolism. Iron homeostasis in yeast can be summarized as four general steps: iron uptake, iron trafficking, iron storage, and iron recycling or removal (Figure 1.2) (5).

1.2.1 Iron Uptake

There are two pathways for iron uptake in *S. cerevisiae*, one is high affinity iron uptake; the other is low affinity iron uptake. Prior to iron uptake, Fre1 and Fre2, which are metal reductases located in the plasma membrane, reduce ferric ions to the more soluble form of ferrous ions. After iron reduction, ferrous ions can be transported through these two different transport systems. In the high affinity pathway, under conditions of iron depletion ferrous ions are first oxidized to ferric ions by Fet3, which is a multicopper oxidase, and are translocated through Ftr1 permease at the surface of the plasma membrane. Under conditions of iron sufficiency ferrous ions can also be transported by Fet4, which has a diverse ability for transporting not only iron but also the other divalent metal ions. In yeast, iron can also be transported into the cytosol by complex formation with low molecular weight compounds, such as siderophores. So far several genes have
been identified as siderophore transporters, including Enb1, Sit1, Arn1, with help of cell wall manoproteins, Fit1, Fit2, and Fit3. Taf1 is also suggested to be a siderophore transporter (5).

Figure 1.2. Iron metabolism and regulation in S. cerevisiae (Adapted from (5)).
In mammalian system, iron internalization by cells is carried out by the well-defined transferrin cycle. Iron-bound transferrin is first recognized by a transferrin receptor on the surface of cell. After binding, it will undergo the process of endocytosis process and follow the formation of endosome. Since iron is very tightly bound to transferrin, with a dissociation constant around $10^{-23}$ M at pH 7.4, the release of iron from transferin can be achieved by a proton pump that decreases the pH inside the endosome. The released iron is then transported into the cytoplasm by divalent metal ion transporter (DMT1). After transporting, iron can be either stored in cytoplasmic ferritin or hemosiderin, or be transported into mitochondria for heme synthesis and iron sulfur cluster assembly. Finally the apo-transferrin and transferrin receptor are recycled to the cell surface for further iron uptake (Figure 1.3) (1).

1.2.2 Iron Trafficking in Cells

Unlike copper, with a well-defined trafficking system, no iron carrier has been identified, after iron is transported into cytosol for transporting iron to the different compartments, such as vacuole, golgi, nucleus, and mitochondrion. Fet5 and Fth1 have been implicated for iron translocation into vacuoles(5). Mrs3/4 have been implicated for iron import in the mitochondrion requiring membrane potential (pmf) for iron translocation and for heme synthesis (6). Mft1/Mft2 has also been implicated for iron import in mitochondrion (7), but it is not required for iron incorporation into heme. The detail mechanism is still unclear. Since iron can’t freely exist in solution, further
investigation of carrier proteins or peptides is necessary. To screen the interaction proteins or peptides of Fet5/Fth1, Mrs3/4, and Mft1/Mft2 maybe the first step toward discovering iron carriers in cytosol.

Figure 1.3 Transferrin cycle for iron uptake in mammalian system (Taken from (1)).
1.2.3 Iron Storage and Recycling

In mammalian systems iron is stored in ferritin, a heteropolymer that contains 24 subunits composed of H(heavy)-chain (Mr ≈ 21,000) and L(light)-chain (Mr ≈ 19,500) components. The mature ferritin shells are able to accommodate up to 4500 iron atoms in a central core. Iron enters this central core as a divalent ion, which is physiologically more soluble than ferric ions and kinetically labile relative to ferric ions. After entering the ferritin core, ferrous ions are subsequently oxidized to stable ferric ions with formation of a crystalline lattice. Two possible mechanisms have been proposed: ferric ions are reduced to ferrous ions by FMN-containing enzyme and released from the ferritin core, or degradation of ferritin subunits results in the iron release. However, the detailed mechanism of iron release from the ferritin core is still unclear (2,8-10). Yeast, unlike mammals, lacks ferritin, and iron is stored in vacuoles for further use. Iron enters vacuole through the Fth1 and Fet5 complex (Figure 1.2). Fth1 is an iron transporter homologue of Ftr1, and Fet5 is an iron oxidase Fet3 on the plasma membrane. Smf3, a vacuolar H+/Fe^{2+}, Mn^{2+} transporter (Figure 1.2) has been suggested for iron release and for further iron usage (5). For plants, iron is stored in plastidic phytoferritin (4).

1.3 Iron Regulation

In mammals iron uptake is controlled at the translational level, however, in \textit{S. cerevisiae} iron uptake is under transcriptional control. In \textit{S. cerevisiae}, the high affinity iron uptake system including Frt1/Fet3 iron uptake complex and Fre1 and Fre2, iron reductase, is regulated by the Aft1 protein (11), which is an iron responsive transcription factor or iron sensor. The molecular weight of Aft1 is around 98 kD. There is a histidine-
rich region at the N-terminus of Aft1, a glutamine-rich region at the C-terminus, and a basic region between 140 and 280 residues containing four cysteine residues that may provide for iron binding during iron regulation. The genes that are involved in iron metabolism are summarized in Table 1.1. Under conditions of iron depletion, Aft1 binds to the upstream region of genes and activates the transcription of iron uptake. The consensus DNA sequence of the Aft1 binding site is PuPyCACCCPy. On the other hand, under iron repletion, iron-bound Aft1 is released from the DNA binding site and downregulates the transcription of iron uptake (Figure 1.2) (5). Surprisingly, low affinity iron uptake is not regulated by Aft1. Recently, the newly discovered Aft1 homologue, Aft2 has been speculated to mediate transcription regulation of Fet4 (12).

In mammals, iron metabolism is regulated by iron-responsive elements (IREs) and iron regulatory proteins (IRPs). IREs are stem-loop structures in the mRNA of iron regulated proteins. The consensus sequence of the stem-loop structure in mRNA is CAGUGN. There are two iron regulatory proteins identified so far, IRP1 and IRP2. IRP1 is a mitochondrial aconitase-like protein, which can form a 4Fe-4S center and has an aconitase activity. During iron repletion, IRP1 will bind to IRE at the 5’ end of ferritin mRNA that will inactivate the expression of ferritin, whereas, IRP1 will bind to five IREs at the 3’ end of the mRNA of transferrin receptor (TfR) that will stabilize mRNA of TfR and result in increasing the expression of TfR. On the other hand, during iron repletion, IRP1 will form a holo 4Fe-4S cluster protein. This will abolish IRP1 binding to the 5’ IRE of ferritin mRNA and 3’ IREs of TfR mRNA. The release of IRP1 from 5’ IRE of ferritin mRNA will activate the expression of ferritin for iron storage, whereas, the
release of IRP1 from the 3’ IREs of TfR mRNA will destabilize the TfR mRNA and result in a decrease both in the expression of TfR and in iron uptake (Figure 1.4) (2).

Figure 1.4. Iron regulation mechanism in mammalian system (Taken from (2)).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>FET3</td>
<td>High affinity iron uptake</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FTR1</td>
<td>High affinity iron permease</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FRE1</td>
<td>Ferric reductase</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FRE2</td>
<td>Ferric reductase</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FTH1</td>
<td>Vacuole iron transport</td>
<td>Vacuole</td>
</tr>
<tr>
<td>FET5</td>
<td>Vacuole iron transport</td>
<td>Vacuole</td>
</tr>
<tr>
<td>FRE3</td>
<td>Siderophore-iron reductase</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FRE4</td>
<td>Siderophore-iron reductase</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FRE5</td>
<td>Ferric reductase</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>FRE6</td>
<td>Ferric reductase</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>ARN1</td>
<td>Siderophore-mediated iron transport</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>ARN2/TAF1</td>
<td>Siderophore-mediated iron transport</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>ARN3/SIT1</td>
<td>Siderophore-mediated iron transport</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>ARN4/ENB1</td>
<td>Siderophore-mediated iron transport</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>ISU1</td>
<td>Iron-sulfur cluster assembly</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>ISU2</td>
<td>Iron-sulfur cluster assembly</td>
<td>Mitochondrial</td>
</tr>
</tbody>
</table>

Table 1.1 Aft1 target genes involving in iron metabolism (Adapted from (5))
1.4 Iron Sulfur Cluster Assembly and Trafficking

Iron-sulfur cluster proteins, which are non-heme iron proteins, are involved in several biochemical processes: aconitase in the citric acid cycle, nitrogenase in nitrogen fixation, cytochrome complex I/II/III in electron transfer of respiration, and IRPs in iron transcriptional regulation. In *S. cerevisiae*, iron-sulfur cluster proteins can be found in the nucleus, cytosol, and mitochondrion. Details for each protein are summarized in Table 1.2 (13). Iron sulfur proteins can contain only one iron center, for example, rubridoxin, or 2Fe-2S cluster, such as ferredoxin in electron transfer, or 4Fe-4S cluster, such as aconitase.

The first reconstituted iron sulfur cluster from clostridial ferredoxin was obtained in vitro in 1966 by Malkin and Rabinowitz (14). Until recently, the mechanism of iron-sulfur cluster assembly was studied only in *Azotobacter vinelandii* for proteins involved in nitrogen fixation. It was suggested that the biosynthesis of iron–sulfur proteins required several assistant components for their maturation. This assembly complex is called the ISC (iron sulfur cluster) machinery. This gene machinery (Figure 1.5) (15) has been identified in several organisms, such as *azotobacter vinelandii*, *E. coli*, and *S. cerevisiae*. The mechanism of iron sulfur cluster assembly has been intensively studied in recent years. The details of this mechanism will be discussed in the following paragraph.

In eukaryotic cells, mitochondria have been proposed as the locus for iron sulfur cluster assembly. There are several key players involved in this process (Figure 1.6), for example, Isu1/2, Isa1/2, Yah, Yfh, Nfs, and molecular chaperone Ssq1 and Jac1. After iron enters the mitochondrion, Isu1/2 or Isa1/2 play a role as scaffold proteins for iron or...
iron sulfur cluster transfer to an apo-protein. NfS provides inorganic sulfide for cluster formation. The process of iron-sulfur cluster formation requires electrons or a reducing source. Yah, which is an adrenodoxin homologue, acquires electron from NADH for ferric ion reduction. Yfh may play a role for iron storage in mitochondria as an iron source. The molecular chaperone Ssq1/Jac1 may assist the iron sulfur cluster formation during this process (13,15). However, the precise mechanism is still unclear and debatable, the additional studies to unveil this process are necessary.

Iron export from mitochondria recently has also been intensively studied. Atm1 from *S. cerevisiae* is located in the mitochondrial inner membrane has been identified as an iron or iron-sulfur exporter. Atm1 (16), a human ABC7 homologue belongs to ABC transporter superfamily that requires ATP hydrolysis for substrate translocation. The introduction of ABC transporters will be discussed detail in the next section. The defect in human ABC7 can cause a hereditary recessive disease called X-link sideroblastic anemia and ataxia (XLSA/A) (17). It also results in accumulation of iron in mitochondria. This phenotype is similar to those defects in iron sulfur cluster assembly machinery except no impairment to mitochondrial iron sulfur proteins is observed, but only to cytosolic iron sulfur proteins. The deletion of YFH, NFS1, ERV1, and GSH1 also show the same phenotype as deletion of ATM1. These suggest all these components may be involved in iron or iron sulfur cluster export in mitochondria and iron sulfur assembly in the cytosol. The detailed function of these genes is summarized in Table 1.3 (13). For heavy metal detoxification in *Schizosaccharomyces pombe*, Hmt1 (18), an ABC type transporter has been discovered for transporting heavy metal into vacuoles. *In vitro* studies show Hmt1 vacuolar vesicles to uptake Cd\(^{2+}\), glutathione, glutathione-conjugates,
apo-phytochelatins and phytochelatin-Cd\(^{2+}\) complexes. Phytochelatin has the general structure \((\gamma\text{-Glu-Cys})_n\text{-Gly}\), where \(n = 2-11\). Furthermore, many ABC transporters can transport either glutathione or glutathione-conjugates across biomembrane (19-24). Together these observations strongly suggest that glutathione may directly or indirectly carry iron or cluster across the mitochondrial membrane to the cytosol. In Chapter 3, the detailed mechanism of iron sulfur assembly in the cytosol will be further discussed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG2</td>
<td>Repair of DNA base damage</td>
<td>Nucleus</td>
</tr>
<tr>
<td>LEU1</td>
<td>Leucine biosynthesis</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>ECM17</td>
<td>Cell wall biogenesis and architecture</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>GLT1</td>
<td>Glutamate biosynthesis</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Rli1</td>
<td>Hypothetical protein</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>IRP1</td>
<td>Iron regulatory protein</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>ACO1</td>
<td>Converts citrate to isocitrate</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>LYS4</td>
<td>Converts homocitrate to homoisocitrate</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>ILV3</td>
<td>Valine and isoleucine biosynthesis</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>YAH1</td>
<td>Adrenodoxin homolog</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>LIP5</td>
<td>Synthesis of alpha-(+)-lipoic acid</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>BIO2</td>
<td>Biotin synthesis</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Complex II</td>
<td>Respiratory chain</td>
<td>Mitochondrial inner membrane</td>
</tr>
<tr>
<td>Complex III</td>
<td>Respiratory chain</td>
<td>Mitochondrial inner membrane</td>
</tr>
</tbody>
</table>

Table 1.2 Summarization of iron sulfur cluster containing protein in \textit{S. cerevisiae} (Summarized from (13)).
Figure 1.5 Gene organization of ISC machinery (Taken from (15)).

Figure 1.6 Iron metabolism in the mitochondrion (13,25).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Phenotype</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM1</td>
<td>Iron/iron-sulfur export</td>
<td>Iron accumulation in mitochondria and impaired iron-sulfur cluster in cytosol and mitochondria</td>
<td>Inner membrane of mitochondria</td>
</tr>
<tr>
<td>YFH</td>
<td>Iron Storage</td>
<td>Iron accumulation in mitochondria and impaired iron-sulfur cluster in cytosol and mitochondria</td>
<td>Mitochondrial matrix</td>
</tr>
<tr>
<td>NFS1</td>
<td>Cysteine desulfurase</td>
<td>Iron accumulation in mitochondria and impaired iron-sulfur cluster in cytosol and mitochondria</td>
<td>Mitochondrial matrix</td>
</tr>
<tr>
<td>Evr1</td>
<td>FAD-linked sulphydryl oxidase</td>
<td>Impaired iron-sulfur cluster in cytosol</td>
<td>Mitochondrial interspace</td>
</tr>
<tr>
<td>GSH1</td>
<td>Catalyzes first step in glutathione biosynthesis</td>
<td>Impaired iron-sulfur cluster in cytosol</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>

Table 1.3 Genes involve in cytosolic iron sulfur cluster maturation and their deletion phenotype (16,24,26-28).
1.5 ABC Transporters

1.5.1 An Introduction to ABC Transporters

ABC (ATP Binding Cassette) transporters (29-31) comprise a large family of membrane proteins, serving either as an importer or exporter. However, there are some exceptions, such as MutS and Rad50 for DNA repair, and the cystic fibrosis transmembrane regulator (CFTR) as a chloride channel. Bacteria especially contain ~ 80 ABC transporters, about 2% of the genome. There are 31 ABC transporters in the Saccharomyces cerevisiae genome that have been discovered as far. For the human, 48 ABC transporters have been discovered, and most of them are exporters involved in detoxification processes. In this ABC superfamily, the ABC transporters possess a large diverse substrate specificity, transporting small molecules like ions, to larger molecules like proteins. Even with this substrate diversity, there is a conserved ATP binding domain, which provides an energy source for transporting substrate across lipid bilayers. An ABC transporter is always composed of four domains; two transmembrane domains and two conserved ATP binding domains. Detailed structural information of ABC transporters will be discussed in the next section.

1.5.2 Structure and Domain Composition

ABC transporters (29) contain four domains, including two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) (Figure 1.7), but there are some exceptions, for example: the sulfonylurea receptor (SUR), MPR1-3 and MRP6-7 with an additional N-terminal transmembrane domain (TMD0). For eukarya, ABC
transporters tend to form a single polypeptide containing four domains or form a half type transporter with one TMD and one NBD (Table 1.4) (29). For bacteria, each domain tends to form as an individual polypeptide (Table 1.4). This may provide an indication that eukaryotic and bacterial ABC transporters possess structural and functional differences, especially for domain interaction.

The transmembrane domains (TMDs) of most ABC transporters span the biomembrane six times. The crystal structure of MsbA (32), which is a member of the MDR family for lipid A transport, has been solved recently. From the structure information, each TMD contains six α-helices. However, the crystal structure of the E. coli ButCD protein (33), which is an ABC transporter mediating vitamin B12 uptake, exhibited a different structure from that of MsbA (32) containing ten α-helices in each transmembrane domain. This difference may suggest why ABC transporters possess a wild variety of substrate specificity. The details of substrate specificity will be discussed in next sub-section.

Unlike transmembrane domains, nucleotide binding domains (NBDs) exhibit a high degree of similarity and identity, with an ATP binding pocket for ATP hydrolysis that provides an energy source for substrate translocation and conformation change during transport. Normally this nucleotide binding domain contains 90-110 amino acid residues. The sequence contains three conserved motifs – Walker A, Walker B, and linker region (Figure 1.8) (30). Walker A and Walker B are primarily for ATP binding and involving in ATP hydrolysis. The linker region is for structural coupling for substrate binding and translocation. Because of the clear differences of
structure and domain organization of ABC transporters between bacteria and eukarya, a detailed analysis of the domain interaction may be required. The detailed analysis of how domain interaction arised will provide complete information oo the transporting mechanism.

Figure 1.7 Structure and domain organization of ABC transporter (Modified from (29)).
Figure 1.8 Nucleotide binding domain and its conserved motifs (Modified from (30)). * means the identical amino acid residue.
1.5.3 Function and Substrate

Because of the flexibility of the transmembrane domain, ABC transporters can transport a wild variety of substrates ranging from small chloride ion, nutrients such as histidine and maltose, to large proteins such as haemolysin (Table 1.4) (29). Most ABC transporters exhibit very high substrate specificity, such as histidine permease, which transports only histidine across the biomembrane. For some other ABC transporters, such as p-glycoprotein and the bacterial homologue LmrA (34), they can transport a wide range of hydrophobic organic drugs across a biomembrane. ABC transporters can function not only as importers, such as histidine permease (35) but also as exporters, such as Atm1p, for substrate translocation.

Table 1.4 Summarization of function, structure organization, and substrate specificity (Adapted from (29)).

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Organism</th>
<th>Organization</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-glycoprotein</td>
<td><em>Homo sapiens</em></td>
<td>(TMD-NBD)2</td>
<td>Hydrophobic drugs</td>
</tr>
<tr>
<td>LmrA</td>
<td><em>Lactococcus lactis</em></td>
<td>2(TMD-NBD)</td>
<td>Hydrophobic drugs</td>
</tr>
<tr>
<td>CFTR</td>
<td><em>Homo sapiens</em></td>
<td>(TMD-NBD)2</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>HylB</td>
<td><em>S. aureus</em></td>
<td>2(TMD-NBD)</td>
<td>Haemolysin</td>
</tr>
<tr>
<td>ABCA4</td>
<td><em>Homo sapiens</em></td>
<td>(TMD-NBD)2</td>
<td>Retinoids</td>
</tr>
<tr>
<td>HisQMP2J</td>
<td><em>S. typhimurium</em></td>
<td>5polypeptides</td>
<td>Histidine</td>
</tr>
<tr>
<td>MalFGK2E</td>
<td><em>E. coli</em></td>
<td>5polypeptides</td>
<td>Maltose</td>
</tr>
<tr>
<td>TAP1/2</td>
<td><em>Homo sapiens</em></td>
<td>2(TMD-NBD)</td>
<td>Antigenic peptides</td>
</tr>
</tbody>
</table>
CHAPTER 2

CHARACTERIZATION OF THE SOLUBLE DOMAIN OF THE ABC7 TYPE TRANSPORTER ATM1

2.1 INTRODUCTION

The ABC (ATP binding cassette) transporters comprise a large family of integral membrane proteins responsible for the ATP-dependent translocation of solutes across biological membranes in both prokaryotes and eukaryotes. ABC transporters are composed of four structural domains: two nucleotide-binding domains (NBD’s), which show a high degree of sequence similarity throughout the family, and two transmembrane domains that typically show six transmembrane spanning helices (36). The structure and function of ABC-type proteins has been intensively studied (37-41). Recently, the first high-resolution X-ray crystal structure of a prokaryotic NBD of histidine permease (HisP) was reported (42) and has provided insight on the structural basis for function in this class of ATP-dependent transporters. In prokaryotic systems these transport complexes are usually constituted of individual subunits, while in the eukaryotic ABC
transporters they are normally formed from a single peptide chain (36), and so a comparison of prokaryotic and eukaryotic systems may yield considerable insight on structure-function correlation’s between these discrete families.

*Saccharomyces cerevisiae* Atm1p is an ABC transporter that is located in the mitochondrial inner membrane and has been implicated in iron-sulfur cluster assembly and maturation in the cytosol (16,43-47). It is a transmembrane spanning protein that is putatively involved in the transfer of iron-sulfur clusters from the mitochondrial matrix to the cytosol (27). In addition to the transmembrane spanning domain the protein also possesses a soluble nucleotide-binding domain that mediates hydrolysis of ATP to ADP. Presumably this ATPase activity is involved in regulating the opening and closing of the channel, and/or driving substrate through the channel. The human homologue of Atm1p, ABC7, displays a very high sequence homology in the soluble nucleotide binding domain. Defects in the human ABC7 gene have been shown to cause a rare type of X-linked sideroblastic anemia associated with cerebellar ataxia (XLSA/A) (17). Similar mutations in Atm1p or ABC7 have been shown to result in the accumulation of high levels of free iron in the mitochondrion and a lack of iron-sulfur clusters in cytosolic proteins. Three other proteins (Bat1, Bat2 and Erv1) have also been suggested to assist this ABC transporter for iron-sulfur cluster translocation (26,48), however, the functional role of Atm1p in mediating iron-sulfur cluster trafficking remains unknown.

Three mitochondrial ABC transporters have thus far been discovered in yeast (45), although the structural and functional properties of these eukaryotic transporters are poorly understood. In this chapter we successfully complete the cloning, purification and characterization of the soluble C-terminal ATP-binding domain of the
Schizosaccharomyces pombe Atm1 (Atm1-C) and make a detailed comparison with the bacterial HisP protein. This provides a starting point for studies of eukaryotic ABC7-type transporters and an initial step toward the understanding of iron-sulfur cluster export mechanisms in mitochondria.

2.2 METHODS AND MATERIALS

2.2.1 Construction of pET21-atm1-C Expression Vector

The soluble C-terminal domain of Atm1 (Atm1-C) was predicted by computer analysis of the transmembrane segment using the program Dense Alignment Surface method (DSA) (49). The nucleotide-binding domain of the prokaryotic His permease (HisP) was found to be similar to that for Atm1-C, and was further characterized by use of the search tool SWISS-MODEL (www.expasy.org/swissmod/SWISS-MODEL.html). The Atm1-C sequence starts from Leu436 of Atm1 and the corresponding gene fragment encoding Atm1-C was PCR amplified by use of the following primers: 5'- GGC CGC CAT ATG CTT AAA GGC GGA TCT ATT CAA TTC G -3' and 5'- CGC GGA TCC TCA TGC ATC TCC GGA TTT ATT CGA TTC -3'. Subsequently, this gene was cloned into pET-21b(+) between the NdeI and BamHI restriction sites. The sequence of expression vector pET21-Atm1-C was finally confirmed by DNA sequence analysis and subsequently transformed into an E. coli BL21 (DE3) host.
2.2.2 Overexpression and Purification of Atm1-C

Expression plasmid pET21-Atm1-C was transformed into the expression host *E. coli* BL2 (DE3) as described elsewhere (50). Cells were grown to an OD600 ~ 0.7 and were then induced with 0.1 mM IPTG at 37 °C for 4 h. After harvesting and washing with 50 mM Tris.HCl pH 7.3, the cells were lysed by sonication with 50 mL of 50 mM Tris.HCl pH 7.3. Since Atm1-C was found to form inclusion bodies, following sonication the pellet was washed in turn with 50 mL of each of 50 mM Tris.HCl, 50 mM Tris.HCl 100mM NaCl, 50 mM Tris.HCl 200mM NaCl, 50 mM Tris.HCl 500mM NaCl, and 50 mM Tris.HCl, 1% Triton X-100 pH 7.3 buffers to remove soluble and membrane-associated proteins. Subsequently, the Atm1-C inclusion body was dissolved in 20 mL solubilization buffer (50 mM Tris.HCl, 100 mM NaCl, 2 M guanidine.HCl, 1 mM ATP, 0.1 mM EDTA pH10.0) at 4 °C for overnight. The denatured solution was centrifuged at 15000 rpm to remove the insoluble pellet. Subsequently the supernatant was diluted ten-fold by slow addition of a pH 10.0 buffer containing 50 mM of Tris.HCl, 100 mM NaCl, 1 mM ATP, and 0.1 mM EDTA at 4 °C. After renaturation, the refolded Atm1-C was concentrated by Amicon ultrafiltration. Pure Atm1-C was obtained following ion exchange chromatography (DE52) and gel filtration (G75) chromatography. For DE52 purification, the column was washed with 500 mL 50 mM TrisOH, pH 10.0, and subsequently the protein was eluted with a gradient from 0 M to 250 mM NaCl in 50 mM TrisOH and 0.1 mM EDTA pH 10.0. The running conditions for the G75 column included an equilibration buffer with 50 mM TrisOH, 100 mM NaCl and 0.1 mM EDTA pH 10.0. The purity of Atm1-C was based on 12% SDS-PAGE electrophoresis analysis.
2.2.3 Determination of the Aggregation State of Atm1-C

The aggregation state of Atm1-C was determined by gel filtration chromatography using a superose12 column on a Pharmacia FPLC system. The experiment was performed in a pH 10.0 buffer containing 100 mM Tris.HCl, 100 mM NaCl and 0.1 mM EDTA. The gel filtration column had previously been calibrated with the FPLC molecular weight standards lysozyme (14.3 kDa), deoxyribonuclease I (31 kDa), pepsin (35 kDa), bovine serum albumin (BSA) (66 kDa), and BSA dimmer (132 kDa). Blue dextrin was used to determine the dead volume. MW’s were determined by plotting log MW of standards vs. Kav where $Kav = (V_e - V_0)/(V_t - V_0)$, $V_e$ = elution volume, $V_0$ = dead volume, and $V_t$ = total column volume.

2.2.4 Fluorescence Determination of ATP and ADP Binding Constant to Atm1-C by Fluorometer

A 500 µL solution of 10 µM Atm1-C in 50 mM Tris.HCl (pH 7.3 or pH 10.0) was titrated with 1 µL aliquots of ATP or ADP (from 5 or 50 mM stocks) in reaction buffers containing 50 mM Tris.HCl (pH 7.3 or pH 10.0). The total volume of titrant added was 20 µL and the effect of the dilution on the emission intensity was negligible. The change in fluorescence emission was monitored by use of a Perkin Elmer LS50B luminescence spectrometer with an excitation wavelength of 280 nm (Tyr and Trp excitation) or 295 nm (Trp excitation) and an emission wavelength of 330 nm or 340nm, respectively. The data was plotted as $\Delta I/I^0$ versus ATP or ADP concentration and fitted by use of the equation $\Delta I/I^0 = \text{const.}[S]/(K_D+[S])$ (51), where $\Delta I = (I^0 - I_{obs})$ and $I_{obs}$ is the observed emission following each addition of titrant with a resulting nucleotide concentration.
defined by [S]. The constant accounts for the fact that the saturating emission is not necessarily zero; that is, the emission is not completely quenched. Corrections for the inner filter effect were made using the equation $I_{corr} = I_{obs} \cdot 10^{A/2}$ (52), where $I_{corr}$ is the corrected emission intensity, $I_{obs}$ the observed intensity, and $A$ is the absorbance at the excitation wavelength.

To confirm the requirement for a structured binding pocket to promote nucleotide binding a control experiment was carried out. A mixture containing 150 µL of 10 µM Atm1-C in 50 mM Tris.HCl and 6 M guanidine.HCl, pH 7.3 was titrated with ATP or ADP in reaction buffer 50 mM Tris.HCl and 6 M guanidine.HCl, pH 7.3, and the variation in emission intensity monitored at 330 nm and 340 nm as described earlier.

2.2.5 ATPase Activity Assay

A 350 µL volume of a reaction mixture containing 30 µM of Atm1-C in an assay buffer consisting of 50 mM Tris.HCl, 100 mM NaCl, and 0.1 mM EDTA (pH 7.3 or pH 10.0) was incubated with 2 mM ATP at 37 °C for 3 min, and the reaction initiated by addition of 50 mM MgCl$_2$ to a final concentration 2 mM. A 50 µL aliquot of the reaction mixture was taken and placed in a tube containing 50 µL of 7.5% SDS. The amount of inorganic phosphate released was determined by a colorimetric assay, using Na$_2$HPO$_4$ as a standard and monitoring the absorbance change at 820 nm (35,53).
The pH profile for the reaction displayed bell-shape behavior and was fit by use of equation 2.1, where \( V_{\text{max}} \) represents the maximal activity under saturating conditions, \( V_{\text{opt}} \) represents the optimum initial velocity with respect to pH, and the pKa’s represent the fitted ionization constants assuming two ionization events.

\[
V_{\text{max}} = \frac{V_{\text{opt}}}{\left( 1 + 10^{\frac{-\text{pH}}{10}} + 10^{\frac{-\text{pKa1}}{10}} + 10^{\frac{-\text{pKa2}}{10}} \right)} \tag{2.1}
\]

2.2.6 Influence of Phospholipids on ATPase Activity

Experiments to examine the phospholipid dependence of the reaction of Atm1-C were performed with 20 µM Atm1-C with 2 mM ATP in a pH 8.0 assay buffer containing 50 mM Tris.HCl, 100 mM NaCl, and 0.1 mM EDTA. The reaction was preincubated on ice for 30 min with 0.04, 0.2, and 1 mg/mL of phosphatidylethanolamine (PE) or cardiolipin and initiated by addition of MgCl\(_2\) to a final concentration 2 mM. Quantitation of the amount and rate of phosphate release has been described in the section describing the ATPase activity assay.

2.2.7 Metal Dependence of the ATPase Activity of Atm1-C

The metal dependence of the reaction was examined with 20 µM Atm1-C and 2 mM ATP in an assay buffer containing 50 mM Tris.HCl, pH 8.0, 100 mM NaCl, and 0.1 mM EDTA. The reaction was initiated by addition of Mg\(_{2+}\), Co\(_{2+}\), or Mn\(_{2+}\) to the desired concentration. Quantitation of the amount and rate of phosphate release has been described in the section describing the ATPase activity assay. For Mg\(_{2+}\) and Co\(_{2+}\), which displayed inhibitory effects at higher [M\(^{2+}\)] equation 2 was used, while the simpler
equation 3 was used for Mn$^{2+}$ which showed no inhibitory behavior, where $k_{cat}$ and $K_M$ are the standard Michaelis constants, $K_D$ is the dissociation constant for metal promoted activity, $K_I$ is the inhibition constant, and other concentration terms are total enzyme, substrate and metal ion concentrations. Equations 2.2 and 2.3 were readily derived from standard equations (54) by assuming a bound metal to be required for activity and a bound metal requirement for inhibition.

\[
V_0 = k_{cat} [E^0] [S^0] K_I K_D [Mg^{2+}]/((1 + K_D [Mg^{2+}]).(K_I([S^0] + K_M) + K_M[Mg^{2+}])) \]  (2.2)
\[
V_0 = k_{cat} [E^0] [S^0] K_D [Mg^{2+}]/((1 + K_D [Mg^{2+}]).([S^0] + K_M)) \]  (2.3)

2.2.8 Circular Dichroism

Circular dichroism spectra were measured on an Aviv model 202 circular dichroism spectrometer. Far-UV CD spectra were acquired with a 0.3-mm path-length cuvette. The concentration of Atm1-C was 10 µM in 20 mM potassium phosphate, pH 7.4 and 100 mM KCl. Spectra acquired at 20 °C were determined per 0.2 nm in triplicate and averaged. Secondary structure quantitation was determined via the self-consistent method (55) with the Dicroprot V2.5 version 5.0 package (56) obtained from www.ibcp.fr. Buffer spectra were always subtracted.

2.2.9 Modeling

The three-dimensional structure of Atm1-C was predicted by use of the SwissModel First Approach Mode. The input sequence of Atm1-C starts from Leu436 of Atm1 with an additional Met at the N-terminus. The lower Blast P(N) limit for template
selection was set to 0.00001. The three-dimensional structure of HisP was also used as the self-input template file for the tertiary structure prediction of Atm1-C. The final model output was a Swiss-PDB viewer project file (57-59).

2.3 RESULTS

2.3.1 Design and Cloning of the Truncated Atm1-C Gene

The amino acid sequence designation of the C-terminal soluble domain of Atm1, Atm1-C, was based both on the use of a tool to search for similar sequences (SWISSMODEL) and from the structure of the sequence-related bacterial protein, HisP. The amino acid comparison of HisP and Atm1-C is shown in Figure 2.1 (60), and the predicted transmembrane spanning region (49) also provided important guidelines for construction of the nucleotide-binding domain of Atm1. Structural information is limited for ABC transporters and HisP is the only representative of this family with a high-resolution structure for the nucleotide-binding domain (42).

2.3.2 Characterization of Atm1-C Aggregation State

Purified Atm1-C was observed to form a white flurry during concentration by Amicon ultrafiltration, especially in the vicinity of the membrane surface. This most likely reflects contact by a membrane-associated domain remaining in this protein. The aggregation state of Atm1-C was determined by use of gel filtration chromatography. The FPLC elution profile (Figure 2.2) showed that most of the expressed Atm1-C was formed as a high molecular weight aggregate with a small amount of dimer. A very
low concentration of monomer was also detected in buffer lacking $\beta$-mercaptoethanol during gel filtration chromatography. However, Figure 2.2 shows that in buffer containing 0.5% $\beta$-mercaptoethanol the relative amount of both the high molecular weight aggregate and the dimeric form were shown to dramatically decrease relative to the amount of monomer. This suggested formation of intermolecular disulfide bonds in Atm1-C as the principal reason for formation of the high molecular weight aggregate and dimer. By use of appropriate standards the molecular weight of monomer was determined by gel filtration as 28.5 kDa, and the molecular weight of dimer was determined as 56.9 kDa. These are similar to the calculated MW’s of 26.9 kDa and 58.9 kDa, respectively. The MW of each eluted fraction was confirmed by SDS-PAGE.
Figure 2.1. Sequence alignment of HisP and Atm1-C: (*) is an identical residue, (:) is a highly conserved residue, and (.) is a conserved residue.
Figure 2.2. FPLC elution profile for Atm1-C. (left) The elution buffer contains no β-mercaptoethanol, and so the eluted fractions show mainly a highly aggregated form (13) and a dimeric Atm1-C form (40). (right) The sample was treated with 0.5% β-mercaptoethanol, and the buffer also contained 0.5% β-mercaptoethanol. The amount of Atm1-C monomer (15) was observed to dramatically increase. By use of appropriate standards (as described in materials and methods) the molecular weight of monomer was determined by gel filtration as 28.5 kDa, and the molecular weight of dimer was determined as 56.9 kDa. These are similar to the calculated MW’s of 26.9 kDa and 58.9 kDa, respectively. The MW of each eluted fraction was confirmed by SDS-PAGE.
2.3.3 ATP and ADP Binding to Atm1-C

There are six Tyr and one Trp in the sequence of Atm1-C. Both residues are electronically excited following excitation at 280 nm, while only Trp is stimulated by 295 nm excitation. Nucleotide binding to Atm1-C can, and does result in a decrease in the combined emission intensity of both Tyr and Trp (Figure 2.3) and the measured K_d’s are summarized in Table 2.1. Control experiments with denatured Atm1-C (Figure 2.3) demonstrate that the change in emission intensity is a consequence of binding rather than a reduction of energy input as a result of the absorption of light by nucleotide. Specifically, the experiments show that there is no binding for either ADP or ATP after accounting for the inner filter effect of the denaturant. Since nucleotide does not absorb at 295 nm an additional control experiment was carried out by monitoring the weaker Trp emission following 295 nm excitation (data not shown). Within error, similar binding parameters were obtained, and so we conclude that the binding affinity that is measured from the change in emission intensity indeed reflects the actual binding between the refolded Atm1-C domain and ATP or ADP. It is important to note, however that these results do not suggest a specific mechanism for quenching, whether directly through energy transfer from specific Tyr or Trp residues, or as a result of conformational changes that influence the polarity and/or solvent accessibility of the environment around such residues.

Our results from monitoring both Tyr and Trp emission indicate that ADP binds more tightly to Atm1-C than ATP binding at both pH 10.0 and 7.3. The binding
affinity at pH 10.0 is lower than that at pH 7.3, consist with more extensive
deprotonation of residues in Atm1-C at higher pH with an increase in repulsion between
Atm1-C and either ATP or ADP.

Figure 2.3. The dissociation constants for nucleotide binding to Atm1-C were
determined by fluorescence quenching (filled circles). The data shown is for ADP
binding with the y-axis representing the extent of emission quenching and the x-axis the
concentration of nucleotide added. The change in fluorescence emission was monitored
by use of a Perkin Elmer LS50B luminescence spectrometer with an excitation
wavelength of 280 nm and an emission wavelength of 330 nm. A mixture containing
500 µL of 10 µM Atm1-C in 50 mM Tris.HCl (pH 7.3) was titrated with 1 µL aliquots
of ADP (5 or 50 mM stocks) in 50 mM Tris.HCl (pH 7.3) reaction buffer. The total
volume of titrant added was 20 µL. The dilution effect of this titration was negligible
and emission intensities were corrected for the inner filter effect. Control experiments
with guanidinium denatured protein (open circles) showed no evidence for nucleotide
binding. A mixture containing 150 µL of 10 µM Atm1-C in 50 mM Tris.HCl and 6 M
guanidine.HCl, pH 8.0 was titrated with 1 µL aliquots of ADP in 50 mM Tris.HCl, 6 M
guanidine.HCl, pH 8.0. Three stock solutions of ADP were used to cover the desired
concentration range (namely, 1 mM, 10 mM, and 100 mM). The total volume of titrant
added is 16 µL. The volume of titrant added compared to the volume of reaction
mixture was relatively high, and so the influence of dilution on the solution emission
was always corrected following appropriate control experiments.
Table 2.1. Nucleotide binding affinity to Atm1-C measured by fluorescence quenching. A 500 µL solution of 10 µM Atm1-C in 50 mM Tris.HCl (pH 7.3 or pH 10.0) was titrated with 1 µL aliquots of ATP or ADP (5 or 50 mM stocks) in reaction buffers containing 50 mM Tris.HCl (pH 7.3 or pH 10.0). The change in emission at 330 nM was fit to the function $\Delta I/I^o = \text{const.}[S]/(K_D+[S])$ and $K_D$ and the constant term evaluated. Errors are estimated at ± 10%.

<table>
<thead>
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<td></td>
<td>const</td>
<td>K (µM)</td>
<td>const</td>
<td>K (µM)</td>
</tr>
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<td>43</td>
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<tr>
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<td>102</td>
<td>0.48</td>
<td>92</td>
</tr>
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</table>
2.3.4 Atm1-C ATPase Activity and Inhibition

The ATPase activity of Atm1-C was determined from the initial rate of phosphate release, which was linear with time over a period of at least 1 h (Figure 2.4). From the resulting Michaelis-Menten plot (Figure 2.5) the $V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ of Atm1-C were measured as $1.822 \, \mu\text{M min}^{-1}$ (or $0.02 \, \mu$ mole min$^{-1}$ mg$^{-1}$), $512.85 \, \mu\text{M}$ and $0.0552 \, \text{min}^{-1}$ respectively. When analyzed by the Hill equation (61), the apparent Hill coefficient, $n_{\text{app}}$, reflecting the number of substrate binding sites per unit of active enzyme was equal to 1.1 ($\pm$ 0.2).

The dependence of ATPase activity on Atm1-C concentration was determined over a range of protein concentration with 2 mM ATP substrate in each reaction. Methods for quantifying the concentration of liberated phosphate were described in the experimental section. A linear relationship between ATPase activity and Atm1-C concentration was observed, suggesting that each subunit of Atm1-C acted as an individual unit for ATP hydrolysis and consistent with the measured Hill coefficient $n_{\text{app}} \sim 1.1$.

The pH dependence of the ATPase activity of Atm1-C was examined between pH 5.5 and 10.0. Figure 2.6 shows the optimal pH for ATP activity to be around 8, indicative of two ionization events involving distinct residues that may influence ATPase activity.

The pK$_a$’s of these two residues have been determined by use of equation 1, and the values of pK$_{a1}$ and pK$_{a2}$ are determined to be 7.3 and 9.3, respectively. The results are consistent with involvement of specific amino acid residues in general acid and general base catalysis of ATP hydrolysis (62).
Figure 2.4. Determination of the ATP hydrolysis rate following the procedure described in the experimental section. The y-axis shows the amount of phosphate being released and the x-axis is the time in minutes.
Figure 2.5. Michaelis-Menten profile of Atm1-C. The measured values of $V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ are 1.822 µM min$^{-1}$, 512.85 µM and 0.0552 min$^{-1}$, respectively. The cooperativity constant $n_{\text{app}}$ was determined to be 1.1 ± 0.2. The solid line is a fit to the Michaelis-Menten equation and the dotted line is a fit to a Hill equation. $V$ is the rate of phosphate release. A 350 µL volume of a reaction mixture containing Atm1-C in an assay buffer consisting of 50 mM Tris.HCl, 100 mM NaCl, and 0.1 mM EDTA pH 8.0 was incubated with varying concentrations of ATP at 37 °C for 3 min, and the reaction initiated by addition of 50 mM MgCl$_2$ to a final concentration 2 mM. A 50 µL aliquot of the reaction mixture was taken and placed in a tube containing 50 µL of 7.5% SDS to determine the amount of free inorganic phosphate. At each concentration of Atm1-C, the rate of reaction was determined by the amount of inorganic phosphate released.
Figure 2.6. Dependence of ATPase activity on pH. A 350 µL volume of a reaction mixture containing 30 µM of Atm1-C in an assay buffer consisting of 50 mM Tris.HCl, 100 mM NaCl, and 0.1 mM EDTA (pH 5.5, 6, 6.6, 7.5, 8.0, 8.5, 9.5, and 10.0) was incubated with 2 mM ATP at 37 °C for 3 min, and the reaction initiated by addition of 50 mM MgCl₂ to a final concentration 2 mM. A 50 µL aliquot of the reaction mixture was taken and placed in a tube containing 50 µL of 7.5% SDS to determine the amount of free inorganic phosphate. At each pH the rate of reaction was determined by quantitating the release of inorganic phosphate.
The influence of phospholipids on ATPase activity was measured by adding phosphatidylethanolamine or cardiolipin to final concentrations of 0.04, 0.2, 1 mg/mL. Neither showed any significant effect on ATPase activity by comparison with control experiments. This result is also consistent with results from concentration dependence experiments that suggested each subunit of Atm1-C to act as an individual unit for ATP hydrolysis.

Experiments to determine the inhibitory influence of several standard ATPase inhibitors was performed with 20 µM Atm1-C in the presence of 2 mM ATP. The reaction was preincubated with 2 mM inhibitor on ice for 30 min and initiated by addition MgCl₂ to a final concentration 2 mM. Vanadate, nitrate, and azide were used to test for P-type ATPase, V-type ATPase, and F-type ATPase inhibition, respectively. None of these showed any effect on enzymatic inhibition. Only ADP had an inhibitory effect on Atm1-C with an IC₅₀ of 10 mM. This number is comparable to that obtained for HisP (~2 mM) (35).

2.3.5 Metal Cofactor Promotion and Inhibition of Atm1-C Activity
The results shown in Figure 2.7 demonstrated that Mg²⁺ and Co²⁺ stimulate ATPase activity to similar extents. The optimum concentration of these two cations is 0.5 mM, although with increasing cation concentration the ATPase activity was found to decrease. Manganous ion was found to best stimulate ATPase activity, especially at higher cation concentration. This result is generally in agreement with observations made with HisP (35), although in the latter case each of Mg²⁺, Mn²⁺, and Co²⁺ were found to be inhibitory at higher concentration, while only Mg²⁺ and Co²⁺ were inhibitory at higher concentration.
for Atm1-C. The inhibitory influence of the metal ion dependence of ATPase activity was analyzed according to the saturation model that has been described in a previous study of metal cofactor promoted ribonuclease H activity (63). In this saturation model, metal ion bound substrate may become inhibitory at high metal ion concentrations, where either the nucleotide substrate or the enzyme may bind additional metal ions. For such a scenario the metal binding constant ($K_M^{2+}$) and inhibition constant ($K_I$) can be determined by equation 2. The results are summarized in Table 2.2 where it is seen that $Co^{2+}$ shows the largest inhibitory effect on ATPase activity. Since $Mn^{2+}$ shows no inhibitory influence over the concentration range used, a simpler hyperbolic binding equation 3 was used.
Figure 2.7. Cation concentration dependence of the ATPase activity of Atm1-C. ○ is Mm$^{2+}$, ■ is Mg$^{2+}$, and ∆ is Co$^{2+}$. The reaction was assayed in a 350 µL volume of a reaction mixture containing 20 µM Atm1-C and 2 mM ATP in an assay buffer, pH 8.0, containing 50 mM Tris.HCl, 100 mM NaCl, and 0.1mM EDTA. The reaction was initiated by addition of Mg$^{2+}$, Co$^{2+}$, or Mn$^{2+}$ to the desired concentration. A 50 µL aliquot of the reaction mixture was taken and placed in a tube containing 50 µL of 7.5% SDS to determine the amount of free inorganic phosphate. At each metal ion concentration the rate of reaction was determined by quantitating the release of inorganic phosphate.

2.3.6 Circular Dichroism and Secondary Structure

The secondary structure of Atm1-C was determined by CD (Figure 2.8), and is composed of 31.2% of α-helix, 22.2% of parallel β-sheet, 8.1% of antiparallel β-sheet, 23.3% of turn, and 10.3% of others. By solution CD spectra the secondary structure of HisP was found to consist of 40% of α-helix and 23% of β-strands (35), whereas the composition calculated from the crystal structure yields of 38% of α-helix and 23% of β-strands (42). This indicates the secondary structure of Atm1-C in solution to be similar to that of HisP in both solution and crystal forms.
The tertiary structure of Atm1-C was modeled using the crystallographically-determined HisP structure as a template. The structural similarity, following refinement, is illustrated in Figure 2.9.

<table>
<thead>
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<th>metal factor</th>
<th>$K_{M^{2+}}$ (mM)</th>
<th>$K_I$ (mM)</th>
</tr>
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<tr>
<td>Mg$^{2+}$</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
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</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>2.1</td>
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Table 2.2. Metal binding constants ($K_{M^{2+}}$) and inhibition constants ($K_I$) for Atm1-C ATP hydrolysis. The metal dependence of the reaction was examined with 20 µM Atm1-C and 2 mM ATP in an assay buffer containing 50 mM Tris.HCl, pH 7.3, 100 mM NaCl, and 0.1 mM EDTA. The reaction was initiated by addition Mg$^{2+}$, Co$^{2+}$, or Mn$^{2+}$ to the desired concentration. Data for Mg$^{2+}$ and Co$^{2+}$ were fit to equation 2 and for Mn$^{2+}$ to equation 3. No inhibition was observed for Mn$^{2+}$ over the concentration range used.
Figure 2.8. Circular dichroism spectrum of a 10 µM solution of Atm1-C in 20 mM potassium phosphate, pH 7.4 and 100 mM KCl. The spectrum was measured in triplicate at 20 °C and 0.2 nm resolution and averaged.

Figure 2.9. Comparison of the crystallographically-determined HisP (top and middle), and the modeled Atm1-C (bottom) structure. The conserved Tyr in the ATP binding pocket is shown, as well as important His and Lys residues discussed in the text (middle and bottom). Asp and Glu residues flanking the likely nucleotide binding domain that might form an inhibitory binding site for metal cofactor are shown (top).
2.4 DISCUSSION

Atm1-C was purified from an inclusion body that was denatured with 2 M guanidinium hydrochloride in pH 10.0 buffer. Refolding was achieved by slow dilution of the denaturant. While in some instances refolded proteins may lose their native conformation or activity, we have demonstrated Atm1-C to return its ATPase activity. Moreover, as described later, CD spectroscopy suggests a similar secondary structure content for the refolded Atm1-C and HisP. Recent crystallographic studies of HisP have shown the protein to maintain an L shaped structure with arms I and II. Arm II was known to interact with membrane domain (42) providing a ready explanation for the observed tendency of Atm1-C to associate with the membrane during Amicon ultrafiltration.

The pH dependence of the ATPase activity implicated both His and Lys residues in promoting catalytic function. Prior modeling of the ATP binding site on the crystallographically defined HisP (42) had suggested a number of critical contacts between the HisP-bound ATP and amino acid side-chains. These included His19, Lys45 and His211 (HisP numbering) (Figure 2.9). The pH profiles for HisP and Atm1-C show a similar bell shape and presumably utilize conserved residues for general acid/base catalysis. Atm1-C lacks the analogue of His19, however His211 is equivalent to His202 for Atm1-C (Figure 8) and is a likely residue represented by the first inflection point of the pH profile. Lys46 in Atm1-C is retained (Figure 2.9) and is the most obvious side-chain to associate with the second inflection point on the pH profile. By analogy to HisP,
Lys46 and His202 are in hydrogen-bonding range of the β- and γ-phosphate oxygens of ATP, which are critical sites for promoting hydrolytic attack and stabilization of the leaving group, respectively.

Only ADP was found to have any significant inhibitory effect on Atm1-C, with a measured IC$_{50}$ of 10 mM. This value is comparable to that found for HisP, with IC$_{50}$ ~ 2 mM (35). This larger IC$_{50}$ value is consistent with our results from ADP and ATP binding (Table 2.1). Vanadate, a P-type ATPase inhibitor, was found to have no effect on Atm1-C at concentrations up to 10 mM. A similar result was also observed for HisP (35), and no phosphorylated intermediate has been identified. No effect was observed for the influence of two distinct phospholipids (PE and cardiolipin) on the ATPase activity of Atm1-C, however, this contrasts with the case of HisP where it was shown that PE had a stimulatory effect on its ATPase activity, while cardiolipin was shown to have an inhibitory effect on ATPase activity. This further supports the idea that the transmembrane domain effects the conformational change in Atm1-C that is required for substrate export and ADP release from the binding pocket.

The results from the metal dependence of Atm1-C ATPase activity are similar to those obtained for HisP with the exception of the manganese data. For HisP the ATPase activity was inhibited at higher concentrations of manganese ions. The inhibitory influence of high metal ion concentration is readily interpretable in terms of two distinct mechanisms. Either the substrate nucleotide binds additional metal ions at higher M$^{2+}$ concentrations and this inhibits substrate binding and/or ATPase activity, or additional metals bind to the enzyme and these inhibit substrate binding and/or ATPase activity. The former is unlikely since the metal substrate interactions would be similar for HisP
and Atm1-C and this does not readily account for the difference in manganese-promoted activity. Inasmuch as both HisP and Atm1-C show a large number of Asp and Glu residues in the vicinity of the ATP binding site (some are shown in Figure 2.9), both conserved and unique, it is likely that some of these form lower affinity metal binding pockets that can bind additional (but inhibitory) metal ion at higher concentrations (Figure 8). Since divalent manganese has the smallest charge-density of the metal ions used it would be expected to bind less tightly. Presumably the secondary metal binding site on HisP, in the vicinity of the ATP binding site, has a higher affinity for Mn\(^{2+}\) than that found on Atm1-C, and so the inhibitory influence is not manifest over the concentration range used.

The dissociation constants for ATP and ADP binding to Atm1-C (Table 2.1) suggest that ADP binds more tightly to Atm1-C than ATP, most likely reflecting the lower negative charge density of the former and consistent with the further decrease in binding affinity observed at higher pH. The numerous carboxylate side chains in the vicinity of the nucleotide-binding domain are the presumed source of the repulsive interactions. The discrepancy between the measured Michaelis constant \(K_M\) (∼ 513 µM) and \(K_D\) (Table 2.1) can be accounted for both by the slow ATPase activity and relatively rapid off-rate for dissociation of ATP, and the influence the repulsive interaction expected between the ATP-complexed divalent metal ion and positively charged Lys 46 (Figure 2.9).

Atm1-C promoted hydrolysis of ATP results in formation of a higher affinity ADP bound form and presumably provides the energy for a structural change that promotes transmembrane transport. Inasmuch as ADP binds more tightly than ATP,
the mechanism of release may involve conformational changes in the membrane-spanning domain, which is missing in Atm1-C. The turnover number \( (k_{\text{cat}}) \) for ATPase activity is found to be lower, relative to that obtained for the bacterial HisP (42); however, a lower activity is commonly observed for eukaryotic trafficking ATPases relative to the prokaryotic systems. For example, the soluble nucleotide binding domain of the cystic fibrosis transmembrane conductance regulator shows a \( k_{\text{cat}} \) of 0.008 s\(^{-1}\) (64) that is comparable to the value of 0.055 min\(^{-1}\) (0.0092 s\(^{-1}\)) that we report here for Atm1-C. The nucleotide binding affinities are also comparable.

Eukaryotic ABC transporters tend to form a single peptide chain, and so the interaction between solutes and loops in the transmembrane domain may promote the structural change in the NBD domain that stimulates both ATP hydrolysis and ADP release. By contrast, prokaryotic ABC transporters are usually composed of separate ATPase and transmembrane spanning domains, and the isolated NBD domains generally demonstrate activities up to an order of magnitude greater than those of the eukaryotic homologues (36). While Atm1 has been implicated in iron-sulfur cluster transport, no cross-linked product has been identified between Atm1-C and potential iron-sulfur cluster proteins in the matrix. Our data suggests that the interaction site for the natural solute is most likely located in the loops of the membrane-bound transporter domain, with solute binding promoting ATP hydrolysis and subsequent structural changes in this domain.

A similarity in secondary structure for HisP and Atm1-C is suggested by comparison of their CD spectra (Figure 2.8 and reference (35)) and may extend to a similarity in tertiary structure (Figure 2.8). Consistent with this idea, our study of protein aggregation state suggests that Atm1-C tends to form a dimer or higher aggregation state,
similar to what is suspected for ABC transporters *in vivo*. However, our study of the concentration dependence of Atm1-C on ATPase activity, and Hill analysis ($n_{app} = 1.1$) demonstrated that there was essentially no cooperativity in ATP hydrolysis. Therefore while the formation of a dimeric state may be required for this ABC transporter to function, no cooperativity is observed between the two ATP binding subunits. This contrasts with the situation for the prokaryotic HisP transporter where a Hill coefficient of $\sim 2$ was determined for both full length (35) and soluble domains (35). Clearly the full cooperative response can operate through the soluble domain for the prokaryotic transporter (and perhaps independently for the distinct transmembrane subunit) but any cooperative response must be mediated through the transmembrane domain in the case of the eukaryotic Atm1 transporter. These results represent an initial step in our efforts to understand the mechanism of this ABC transporter. Future studies will be directed toward full length Atm1.
CHAPTER 3

CHARACTERIZATION AND PURIFICATION OF THE FULL LENGTH VERSION OF THE ABC7 TYPE TRANSPORTER ATM1

3.1 INTRODUCTION

The ABC (ATP binding cassette) transporters comprise a large family of integral membrane proteins responsible for the ATP-dependent translocation of solutes across biological membranes in both prokaryotes and eukaryotes. ABC transporters are composed of four structural domains: two nucleotide-binding domains (NBD’s) that show a high degree of sequence similarity throughout the family, and two transmembrane domains, each of which usually spans the membrane six times (36). Both the function and structure of this ABC family has been intensively studied (37-41). Recently, the first crystallographic characterization of a transmembrane ABC protein, namely the Escherichia coli lipid flippase MsbA was reported (65) and provided much insight on the structure and function of these ATP-dependent transporters. In prokaryotic systems these transport complexes are usually constituted of individual subunits, while in the eukaryotic ABC transporters they are normally formed from a single peptide chain (36).
Accordingly, comparison of such proteins from prokaryote and eukaryote sources may yield considerable insight on structure-function correlations between these discrete families.

*Saccharomyces cerevisiae* Atm1p is an ABC transporter that is located in the mitochondrial inner membrane and has been implicated in iron-sulfur cluster assembly and maturation in the cytosol (16,43-47). It is a transmembrane spanning protein that is putatively involved in the transfer of iron-sulfur clusters from the mitochondrial matrix to the cytosol (27). In addition to the transmembrane spanning domain the protein also possesses a soluble nucleotide-binding domain that mediates hydrolysis of ATP to ADP. Presumably this ATPase activity is involved in regulating the opening and closing of the channel, and/or driving substrate through the channel. The human homologue of Atm1p, ABC7, displays a very high sequence homology in the soluble nucleotide binding domain. Defects in the human ABC7 gene have been shown to cause a rare type of X-linked sideroblastic anemia associated with cerebellar ataxia (XLSA/A) (17,66-69). Similar mutations in Atm1p or ABC7 have been shown to result in the accumulation of high levels of free iron in the mitochondrion and a lack of iron-sulfur clusters in cytosolic proteins (66-68). Three other proteins (Bat1, Bat2 and Erv1) have also been suggested to assist this ABC transporter for iron-sulfur cluster translocation (26,48). Koeller, DM etc. (47,70) proposed that Atm1 may be involved in iron export and recently they identified MDL1 a mitochondrial peptide exporter as a high copy suppressor of ATM1 in resistance to oxidative stress. However, the functional role of Atm1p in mediating iron-sulfur cluster trafficking remains unknown. In the previous chapter, concerning Atm1-C from *Schizosaccharomyces pombe* (71), we suggested that the soluble domain of this ABC
transporter acts as an individual when it transports substrate across a membrane and the transmembrane domain of this transporter is also required for substrate export. The results from this study suggested the transmembrane domain to be responsible for substrate recognition. To understand the role of Atm1p for maturation of cytosolic proteins and the involvement in the mechanism of mitochondrial iron export, it is necessary to characterize the function of Atm1p in vitro. In this work, we not only describe the first purification of a full length of ABC7 type of ABC transporter, but also provide evidence that the transmembrane domain interaction of this transporter is essential for substrate export.

3.2 METHODS & MATERIALS

3.2.1 Strains, Plasmids, Assay, and Materials

The TOP10F’ E. coli strain, INVSc1 yeast host strain, pYES2 yeast expression vector, and all restriction enzymes were obtained from Invitrogen (Carlsbad, CA). Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). The DNA purification kit and Ni-NTA resin were purchased from Qiagen (Valencia, CA). The E.coli vector pET21b(+) was obtained from Novagen (Madison, WI). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The Immun-Blot Assay and Bradford Assay kits were from Bio-Rad (Hercules, CA). Superoxide-12 resins were obtained from Amersham Biosciences (Piscataway, NJ). The anti-His6-tag antibody was from Santa Cruz (Santa Cruz, CA). The Bradford assay was used to quantitate protein based on Bio-Rad protein assays user’s manual.
3.2.2 Construction of pYES2-Atm1p-K(His)

The full length *atm1p* gene lacking the stop codon was amplified from the isolated chromosomal DNA of *Saccharomyces cerevisiae* by PCR with the designed primers 5’-CCGGAATTCATGGTGGTTCTTCCCAAAGATGTCCTG-3’ and 5’-CCGCTCGAGTAGTTCTTGCTGGTCTTTTAGTTC-3’. Subsequently the *atm1p* gene was cloned into pET21b(+) between the EcoRI and XhoI sites. The resultant plasmid was designated pET21(b)-*atm1p*. To incorporate the Kozak sequence and a HindIII restriction site at beginning of the sequence the designed primers, 5’-TATGAAGCTTACCATGGAGG-3’ and 5’-AATTCCTCCATGGTAAGCTTCA-3’, were cloned between the NdeI and EcoRI sites. The resultant plasmid was designated pET21(b)-*atm1p*-k. Subsequently pET21(b)-*atm1p*-k was restriction digested with HindIII and XhoI and the *atm1p* gene containing the Kozak sequence was cloned into pYES2 between the HindIII and XhoI sites. The resultant plasmid was designated pYES2-*atm1p*-k. Finally, the C-terminal His6-tag was introduced with the designed primers 5’-TCGAGCACCACCACCACCACCACCACCCTGAAGCTT-3’ and CTAGAAAGCTTTTCAGTGGTGGTGGGTGGTGTC-3’ between the XhoI and XbaI restriction sites. The resultant plasmid was designated pYES2-*atm1p*-k-his (Scheme I).

3.2.3 Transformation of pYES2-*atm1p*-k-his into Yeast Strain INVSc1

The yeast strain, INVSc1 and yeast expression vector, pYES2, were purchased from Invitrogen. The lithium acetate method (72-75) was used for plasmid transformation in yeast. Yeast extract peptone dextrose (YPD) medium was used for yeast growth. Following the detailed protocol described in the Invitrogen pYES2 user manual.
3.2.4 Overexpression of pYES2-\textit{atmlp-k-his} in Strain INVScl

For a small scale expression an overnight culture was inoculated and was diluted into 100 mL synthetic minimal defined medium containing 2% raffanose as a carbon source and all amino acids excluding uracil. The cells were grown at 30 °C to an OD at 600 nm around 0.7, and then were induced by addition of 2 g of galactose into the medium while shaking at 300 rpm for 18 hours. Finally, the cells were harvested at 5000 rpm and washed with 50 mL cold water before storing at -80 °C. For a large scale expression, an overnight culture was inoculated and was diluted into 1 L minimal medium containing 2% galactose as carbon source and all amino acids excluding uracil. The cells were grown at 30 °C for 24 h.
Scheme 3.1 Construction of expression vector of pYES2-Atm1p-K(His).
3.2.5 Purification of Atm1p (His) from INVSc1 (76,77):

The cells were first suspended in 30 mL bufferA containing 100 mM Tris-SO₄, pH 9.4 and 10 mM DTT and incubated at 30 °C for 5 min with gentle shaking. Then the cells were centrifuged at 5000 rpm for 5 min and the pellet was washed with 40 mL buffer B containing 1.2 M sorbitol and 20 mM potassium phosphate, pH 7.4. A zymolase solution (2 mg/g cells) was added, 0.6 mg/mL in 10 mL buffer B, incubated at 30 °C for 3 h, and the spheroplasts collected by centrifugation at 5000 rpm for 5 min. The pellet was then suspended in 40 mL ice-cold buffer B and centrifuged. The spheroplasts were lysed in 40 mL buffer C containing 0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4, and 1 mM PMSF and transferred to a 40 mL Dounce homogenizer where the cells were broken by use of a tight fitting glass piston for 20 strokes. The supernatant was transferred to another tube and centrifuged at 5000 rpm for 5 min. The result supernatant was collect and centrifuged at 15000 rpm for 30 min to collect the crude mitochondrial portion. The crude mitochondria were solubilized by vertexing in 20 mL of loading buffer D containing 20 mM Tris-HCl pH 7.9, 500 mM NaCl, and 5% triton X-100 and incubated at 4 °C for 30 min with stirring. After centrifuging at 15000 rpm for 30 min the supernatant was diluted with 40 mL loading buffer D lacking of triton X-100. The supernatant was loaded onto a pre-equilibrated Ni-NTA column and then washed with 40 mL wash buffer E containing 20 mM Tris-HCl pH 7.9, 500 mM NaCl, 20 mM imidazole, and 0.4% CHAPS. Finally, the protein was eluted with 40 mL elution buffer F containing 20 mM Tris-HCl pH 7.9, 500 mM NaCl, 500 mM imidazole, and 0.4% CHAPS.
3.2.6 FPLC – Gel Filtration Chromatography

Atm1p was further purified by FPLC gel filtration with a Superose-12 column (HR 16/50) at a flow rate of 0.4 ml/min with the running buffer containing 20 mM Tris-HCl pH 7.9, 100 mM NaCl, and 0.2% sodium cholate.

3.2.7 Immunoblot

The identity of the FPLC purified Atm1p-His was confirmed by running a 12% SDS-PAGE and using a dot-blotting method. An Immun-Blot assay kit was purchased from Bio-Rad. The first antibody was an anti-His$_6$-tag antibody from goat. The second antibody conjugate was an anti-goat IgG with alkaline phosphatase. First 1 µL of purified Atm1p was applied onto a nitrocellulose membrane and until dry before proceeding to the bloking step. Then the membrane containing Atm1p was incubated in the blocking solution containing 3% of gelatin in 20 mM Tris.HCl, 500 mM NaCl, pH 7.5 for one hour with gentle shaking at RT. After incubation the membrane was washed with washing buffer containing 20 mM Tris.HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5 for 10 min with gentle agitation. Then the membrane was incubated with the first antibody solution containing 1% gelatin, 20 mM Tris.HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5 for overnight at RT. After overnight incubation the membrane was washed twice with buffer containing 20 mM Tris.HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5 at RT before incubated with the second antibody in the antibody solution for 2 hours at RT. To remove the excess antibody, the membrane was then washed twice with washing buffer containing 20 mM Tris.HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5 at RT. Finally the color development of the membrane was obtained after around 10 min with incubation
with the color development buffer and then washed with distilled water to remove the
color development buffer. The detail procedure has been described in Bio-Rad Immun-
Blot assay kit manual.

3.2.8 ATPase Activity Assay

A 350 µL volume of a reaction mixture containing 10 µM of Atm1p in an assay
buffer consisting of 50 mM Tris.HCl, 100 mM NaCl, 0.2% sodium cholate and 0.1 mM
EDTA pH 7.9 was pre-incubated with 2 mM ATP at 37 °C for 3 min, and the reaction
initiated by addition of 50 mM MgCl₂ to a final concentration 1 mM. A 50 µL aliquot of
the reaction mixture was taken and placed in a tube containing 50 µL of 7.5% SDS. The
amount of inorganic phosphate released was determined by a colorimetric assay (53),
using Na₂HPO₄ as a standard and monitoring the absorbance change at 850 nm by use of
a Perkin Elmer Lambda 6 spectrophotometer.

3.2.9 The Influence of Phospholipid on ATPase Activity

The phospholipid dependent reaction of Atm1p was performed in 10 µM Atm1p
with 2 mM ATP in assay buffer pH 7.9 containing 50 mM Tris.HCl, 100 mM NaCl, 0.2%
sodium cholate and 0.1mM EDTA. The reaction was pre-incubated with 0.03, 0.1, and 1
mg/mL of phosphatidylethanolamine (PE), cardiolipin, and crude PC on ice for 30 min
and initiated by addition of MgCl₂ to a final concentration 1 mM. Quantitation of the
amount and rate of phosphate release has been described in the section describing the
ATPase activity assay.
3.2.10 NTPase Activity Assay

The NTPase activity of Atm1p was performed in 10 μM Atm1p with 2 mM NTP in assay buffer pH 7.9 containing 50 mM Tris.HCl, 100 mM NaCl, 0.2% sodium cholate and 0.1 mM EDTA. The reaction was pre-incubated at 37 °C for 3 min and then initiated by addition of MgCl₂ to a final concentration 1 mM. Quantitation of the amount and rate of phosphate release has been described in the section describing the ATPase activity assay.

3.2.11 The Metal Dependence of the ATPase Activity of Atm1p

The metal dependence of the reaction was examined with 10 μM Atm1p with 2 mM ATP in an assay buffer pH 7.9 containing 50 mM Tris.HCl, 100 mM NaCl, 0.2% sodium cholate and 0.1mM EDTA. The reaction was initiated by addition Mg²⁺, Co²⁺, or Mn²⁺ to the desired concentration. Quantitation of the amount and rate of phosphate release has been described in the section describing the ATPase activity assay.

3.2.12 The Influence of Inhibitors on ATPase Activity

A reaction containing 10 μM Atm1p with 2 mM ATP in assay buffer pH 7.9 containing 50 mM Tris.HCl, 100 mM NaCl, 0.2% sodium cholate and 0.1mM EDTA was pre-incubated with different inhibitors (sodium azide, potassium nitrate, sodium thiocynate, sodium vanadate, and adenine diphosphate) to a final concentration of 10 mM on ice for 30 min. The reactions were initiated by addition of MgCl₂ to a final concentration 1 mM. Quantitation of the amount and rate of phosphate release has been described in the section describing the ATPase activity assay.
3.2.13 Circular Dichroism Spectroscopy

Circular dichroism spectra were measured on an Aviv model 202 circular dichroism spectrometer. Far-UV CD spectra were acquired with a 0.3-mm path-length cuvette. Concentration of Atm1p was 10 μM in 20 mM potassium phosphate, pH 7.4, 100 mM KCl and 0.2% sodium cholate. Spectra acquired at 20 °C were determined per 0.2 nm in triplicate and averaged. Secondary structure quantitation was determined via the K2D (78,79) obtained from http://www.embl-heidelberg.de/~andrade/k2d.html. Buffer spectra were always subtracted.

3.2.14 Preparation of Liposomes

Liposomes of each phospholipid were prepared by a sonication method according to literature procedure (80). The concentration of each liposome was prepared to be 50 mg/mL as a stock. The phospholipids were suspended in buffer by vortex mixing and the resulting lipids dispersion was sonicated by a probe sonicator (4 mm tip diameter at 20% power) 10 seconds each time at 4 °C until clear. For each reaction, the 50 mg/mL stock was used and diluted to the desired concentration with reaction buffer.

3.2.15 Fluorescence Quenching (81,82)

The samples for acrylamide quenching are prepared by mixing 9.64 μg of Atm1p in 0.05% triton x-100, 20 mM Hepes, 150 mM NaCl pH 7.0 to a crude soybean L-α-phosphatidylcholine in 20 Hepes, 150 mM NaCl pH 7.0 to a final volume of 1 mL. The final concentration of crude soybean L-α-phosphatidylcholine is 180 μg/mL. The acrylamide quenching experiments are carried out by adding 5 μL of 3 M acrylamide
stock solution into a reaction. The concentration range of acrylamide is from 0 to 87 µM. The change in fluorescence emission at 334 nm of Atm1p with different substrates was monitored by use of a Perkin Elmer LS50B luminescence spectrometer with an excitation wavelength 290 nm at room temperature. The final concentration of different substrates is 1 mM. The acrylamide quenching results are plotted according to Stern-Volmer equation 3.2.1:

\[
\frac{F_0}{F} = 1 + K_{SV}[Q]
\]  

(3.2.1)

Where \(F_0\) and \(F\) are the fluorescent intensity of Atm1p in absence and presence of acrylamide, respectively, \(K_{SV}\) is the Stern-Volmer constant, and \([Q]\) is the concentration of acrylamide.

3.2.16 Preparation of Vesicle Encapsulated Carboxyfluorescein (80,83)

A 1 mg crude soybean L-\(\alpha\)-phosphatidylcholine in 150 µL of 20 mM sodium citrate, 150 mM NaCl pH5.0 was mixed with 5,6-carboxyfluorescien to a final concentration of 80 mM. The resulting mixture was first sonicated by a using probe sonicator with 20% power for 10 seconds each time until clear and then following with bath-type sonicator under argon for 30 min at RT. Unencapsulated carboxyfluorescien was removed by passing through a Sephadex G-100 gel filtration column (2x15 cm) pre-equilibrated with 20 mM Hepes, 150 mM NaCl pH7.0. The vesicle encapsulated with carboxyfluorescein was collected around 1 mL right after void volume. The pH gradient and vesicle were stable in the time-frame of the experiment.
3.2.17 Preparation of Vesicle Encapsulated Phen Green (80,83,84)

A 6 mg crude soybean L-α-phosphatidylcholine in 300 µL of 20 mM Hepes, 150 mM NaCl pH7.0 was mixed with Phen Green to a final concentration of 50 µM. The resulting mixture was first sonicated by using probe sonicator with 20% power 10 seconds each time until clear and then following with a bath-type sonicator under argon for 15 min at RT. The reconstituted vesicle was obtained by adding 50 µg of purified Atm1p into the mixture and sitting on ice for 45 min. Unencapsulated Phen Green was removed by passing through a Sephadex G-100 gel filtration column (2x15 cm) pre-equilibrated with 20 mM Hepes, 150 mM NaCl pH7.0. The vesicle encapsulated Phen Green was collected around 1 mL right after void volume. The control vesicle lacking Atm1p reconstituted was also prepared as the same procedure.

3.2.18 Atm1p Reconstitution, Substrates Titration, and Carboxyfluorescien Release (80,84)

A 150 µL of vesicle encapsulated carboxyfluorescein was placed in a fluorescent quartz cuvette at 22 or 37 °C as indicated. The change of fluorescence signal from carboxyfluorescien was monitored as a function of time in Perkin Elmer LS50B luminescence spectrometer with an excitation wavelength 490 nm and an emission wavelength 520 nm. The titration experiment was performed by adding 1 µg of purified Atm1p into reaction mixture. Atm1p reconstitution process can be monitored by fluorescent change at 520 nm and was accomplished within 10 min time-frame. After Atm1p reconstituted into vesicles, different substrates were added into the reconstituted
vesicles reaction mixture to a final concentration of 0.64 μM. The change of fluorescence signal from carboxyfluorescien was monitored as a function of time at 520 nm. The concentration of each stock solution (ATP, ADP, Mg\(^{2+}\), and VO\(_3\)\(^-\)) was 100 mM.

3.2.19 Transporting Assay (84)

A 150 mL of vesicle encapsulated Phen Green was placed in a fluorescent quartz cuvette at 25 °C. A final concentration of 4.8 mM of DPX was added to the reaction mixture to quench the fluorescence of the external PG outside of vesicles. The change of fluorescence signal from Phen Green was monitored as a function of time in Perkin Elmer LS50B luminescence spectrometer with an excitation wavelength 507 nm and an emission wavelength 532 nm. The reaction was initiated by the addition of different concentration of substrates. The detail will describe in result section and figure legend.

3.3 RESULTS

3.3.1 Cloning, Expression and Purification of Atm1p

The expression and purification of membrane proteins is usually a challenging task as a result of the hydrophobicity of the transmembrane domain; especially when expressed in bacterial hosts. While there are some successful examples of membrane protein expression in *E. coli*, most of those proteins are from prokaryotes (85). Recent reports have described the use of a yeast expression system to over-express eukaryotic membrane proteins at reasonable levels when expression was found difficult in *E. coli* (86). In this work, we utilize a yeast expression system to overcome this problem and
obtain the full length atm1p, a mitochondrial membrane protein that has been implicated in iron-sulfur cluster maturation in the cytosol. The pYES2 nonfusion vector was used for cloning. This vector contains a Kozak translation initiation sequence, an ATG initiation codon, and a HindIII restriction site that were introduced at the initial sequence of atm1p for proper initiation of translation. A His$_6$-tag was then added at the C-terminus for purification purposes, and an extra HindIII restriction site was also introduced for a facile digestion assay. The sequence of pYES2-atm1p-k was confirmed by automated DNA sequencing. Overexpression was carried out in yeast expression host strain INVSc1, which has previously shown reasonable expression levels. Since the mitochondrial targeting pre-sequence is still included in this expression construct, the overexpressed Atm1p is believed to locate in the inner mitochondrial membrane. The purification strategy for yeast expressed Atm1p was to concentrate Atm1p by first isolating the yeast mitochondria in order to separate Atm1p from the other cytosolic proteins before application to a Ni-NTA column. After Ni-NTA column purification, the purity of Atm1p was greater than 80% based on the 12% SDS-PAGE electrophoresis (Figure 3.1-a). Pure Atm1p (> 90%) finally was obtained by passing through FPLC – gel filtration chromatography. The total yield of Atm1p from 3 g of wet yeast cells is around 1 mg of protein. The confirmation of Atm1p was carried out the dot-blotting method and 12% SDS-PAGE (Figure 3.1-a,b). The purified Atm1p in 50 mM TrisHCl pH 7.9, 100 mM NaCl, 0.1 mM EDTA, and 0.2% sodium cholate was stored at 4 °C up to one week with a minor loss around 10% of protein, as determined by Bradford assay.
Figure 3.1. SDS-PAGE of Atm1p. The SDS-PAGE was used is a 12% vertical gel. (a) In lane 1, the zymolase digest yeast cells were solubilized in loading buffer. Lane 2 is the isolated crude mitochondria with over-expressed Atm1p. Lane 3 is Atm1p, which was purified from Ni-NTA chromatography. Lane 4 is Atm1p, which was further purified from FPLC-gel filtration chromatography. (b) The dot-immunoblotting was performed. The purified Atm1p was first applied to membrane and following the binding with first antibody(anti-His) and second antibody with alkaline phosphatase.
3.3.2 Influence of Inhibitors on ATPase Activity

There are three different types of ATPases, including V-type, F-type, and P-type ATPases. Nitrate and thiocynate are thought to be inhibitors of V-type ATPase. Azide and vanadate are inhibitors for F-type and P-type ATPase, respectively. ADP is an inherent inhibitor for ATPase. To determine the ATPase classification for Atm1p these inhibitors were examined. Each of the inhibitors was added to a final concentration of 10 mM. The percentage of ATPase activity was determined by comparison to the control reaction. Sodium vanadate shows only 13% activity, and ADP has 0% activity. This result suggests that Atm1p is a P-type ATPase. The measured IC$_{50}$’s for vanadate and ADP are 4 µM and 0.9 mM, respectively (Figure 3.2).

3.3.3 Kinetics of Atm1p

From the resulting Michaelis-Menten plot (Figure 3.3) the $V_{max}$, $K_M$ and $k_{cat}$ of Atm1p were measured as 8.69 µM/min, 106.98 µM and 1.241 min$^{-1}$ respectively. When the plot was analyzed by the Hill equation, the $n_{app}$, which is the Hill coefficient, or the number of substrate binding sites per molecule of enzyme, was equal to 1.4 (± 0.2). The concentration dependence of the ATPase activity of Atm1p shows a nonlinear relationship between activity and enzyme concentration. This suggests cooperativity between Atm1p for ATP hydrolysis. The hydrolysis of first ATP will affect the second ATP hydrolysis if the enzyme forms a dimer.
3.3.4 The Influence of NTP’s on the ATPase Activity of Atm1p

To test the influence of various NTPs on the enzymatic activity of Atm1p, ATP, CTP, GTP, and UTP were used to measure this effect. The result of these NTPase activity (Figure 3.4) shows that the order of NTPase activity is GTP>CTP>ATP>UTP.

![Figure 3.2. Effect of the different inhibitors on ATPase activity of Atm1p. The activity of Atm1p was assayed in buffer, described in experimental section. The reaction rate was linearly determined by measuring inorganic phosphate every two minutes in ten minutes. The relative rate was calculated based on the control experiment, which has Atm1p on assay without any inhibitor. The rate of control experiment was set to 100%.](image)
Figure 3.3. Michaelis-Menten profile of Atm1p. The measured values of $V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ are $8.69 \, \mu M/min$, $106.98 \, \mu M$ and $1.241 \, \text{min}^{-1}$, respectively. The cooperative constant $n_{\text{app}}$ is determined to be $1.4 \, (\pm 0.2)$. The solid line is a fit to the Michaelis-Menten equation and the dotted line is a fit to a Hill equation.
Figure 3.4. Effect of the different NTP on ATPase activity of Atm1p. The activity of Atm1p was assayed in buffer, described in experimental section. The reaction rate was linearly determined by measuring inorganic phosphate every two minutes in ten minutes. The highest rate (GTP) was set to 100%. The relative rate of other NTPs was calculated compared to the rate from GTP.
3.3.5 The Influence of Divalent Cations on ATPase Activity of Atm1p

To test the influence of divalent cations on the enzymatic activity of Atm1p, Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ were used to measure this effect. The result of these NTPase activity shows that Mn$^{2+}$ is the best stimulator with 4 mM metal ion concentration. Mg$^{2+}$ has the maximum activity at concentration of 1 mM. Co$^{2+}$ shows it is a poor enzymatic stimulator compared to Mn$^{2+}$ and Mg$^{2+}$ with maximum concentration of 4 mM (Figure 3.5).

3.3.6 The Influence of Phospholipids on the ATPase Activity of Atm1p

Crude PC and 0.1 mg/mL of cardiolipin showed a stimulatory effect on the ATPase activity of Atm1p. At concentrations of 1 and 0.03 mg/mL of cardiolipin, and at all concentrations of PE, the ATPase activity of Atm1p is inhibited to a different extent. The result of these phospholipids on the inhibition of ATPase activity of Atm1p is summarized on Table 3.1.

3.3.7 Circular Dichroism Spectroscopy

The buffer used for circular dichroism measurement contains 20 mM potassium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.2% sodium cholate, which has very strong absorbance between 180 and 200 nm. Only the spectrum between 200 and 300 nm are useful for secondary structure determination (Figure 3.6). K2D, which is a web-based program for secondary structure prediction from CD spectrum ranging from 200 to 241
nm, was used for determining the secondary structure of Atm1p. The predicted secondary structure of Atm1p is composed of 40% of α-helix, 17% of β-sheet, and 44% of random coil.

Figure 3.5. Cation concentration dependence of the ATPase activity of Atm1p. ○ is Mn$^{2+}$, Δ is Mg$^{2+}$, and ◊ is Co$^{2+}$. The activity of Atm1p was assayed as described in the experimental section. Each reaction rate was determined from the initial velocity by measuring inorganic phosphate production every two minutes over a period of ten minutes. The reaction was initiated by addition of 100 mM cation stock solution to a desired cation concentration.
Table 3.1. Effect of phospholipids on the ATPase activity of Atm1p. The activity of Atm1p was assayed as described in the experimental section. The reaction rate was linearly determined by measuring inorganic phosphate every two minutes over a period of ten minutes. The relative rate was calculated based on the control experiment, which used Atm1p in the assay without any phospholipid. The rate of control experiment was set to 100%.
Figure 3.6. Circular dichroism spectrum of a 10 µM solution of Atm1p in 20 mM potassium phosphate, pH 7.4, 100 mM KCl, and 0.2% sodium cholate. The spectrum was measured in triplicate at 20 °C and 0.2 nm resolution and averaged.

3.3.8 Determination of Conformation Change by Fluorescence Quenching

The reconstituted Atm1p liposomes with different substrates were titrated with acrylamide stock solution. The intrinsic tryptophan fluorescent change by acrylamide quenching was monitored as a function of acrylamide concentration. Linear fitting, based on Stern-Volmer equation, gives rise to a Stern-Volmer constant ($K_{SV}$) (Figure 3.7). The
Stern-Volmer constant ($K_{SV}$) represents the accessibility of fluorophore to quencher. A comparison with Stern-Volmer constants ($K_{SV}$) of different bound substrates Atm1p provides the conformational relationship between the different bound forms of Atm1p. In this experiment, the excited wavelength at 290 nm was used to avoid the influence from acrylamide. The maximum acrylamide concentration was around 87 $\mu$M. It has been shown in the previous study by Sonveaux N. etc. (82) that acrylamide concentrations over 80 $\mu$M results in a dominant static quenching that gives rise to a nonlinear relationship at higher acrylamide concentration. The calculated $K_{SV}$ is summarized on Table3.2. The results indicated that $K_{SV}$ for both Mg$^{2+}$ADP and vanadate-induced ADP trapped in the binding site of Atm1p show the biggest deviation from that of the non-substrate bound Atm1p. This also suggested that Mg$^{2+}$ADP-bound Atm1p has the biggest conformational change compared to the other substrate bound form of Atm1p. The small change in $K_{SV}$ of ATP, AMP-PNP, and ADP+$P_{i}$ bound Atm1p indicated there is only a slight structural change compared to a none substrate bound Atm1p.
Figure 3.7 Stern-Volmer plots of Trp fluorescence quenching by acrylamide in the absence and presence of substrates. ■ is non-substrate bound Atm1p; ○ is Mg\(^{2+}\)AMP-PNP bound Atm1p; ● is ATP bound Atm1p; ▲ is ADP bound Atm1p; △ is Mg\(^{2+}\)ADP and VO\(_3^–\) bound Atm1p.
<table>
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<th>Ligand</th>
<th>$K_{SV}$(mM$^{-1}$)</th>
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<tr>
<td>none</td>
<td>7.36 ± 0.21</td>
</tr>
<tr>
<td>MgATP</td>
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<tr>
<td>MgAMP-PNP</td>
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<tr>
<td>MgADP and P$_i$</td>
<td>6.58 ± 0.39</td>
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<tr>
<td>MgADP</td>
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</tr>
<tr>
<td>MgADP and VO$_3^-$</td>
<td>5.24 ± 0.22</td>
</tr>
</tbody>
</table>

Table 3.2 Stern-Volmer constants of Trp fluorescence quenching by acrylamide in the absence and presence of substrates.
3.3.9 Determination of ATPase Catalytic Cycle

Crude soybean L-α-phosphatidylcholine was used for liposomes entrapped with carboxyfluorescien, because of its low cost and formation a tightly sealed vesicle (83). Vesicles with encapsulated carboxyfluorescien were first reconstituted with Atm1p in buffer containing 0.2% sodium cholate. During the reconstitution process, the formation of channel will cause liposome leakage, and this will increase the fluorescent intensity at 520 nm by increasing pH inside vesicles. This reconstitution process was accomplished within 10 min. The control experiment was also performed by addition of the same amount of buffer containing 0.2% sodium cholate. There is no fluorescence change due to the addition of detergent. Leakage is therefore unlikely from detergent destabilization of vesicles. After reconstitution, the Atm1p reconstituted vesicles were titrated with the different substrates including ATP, ADP, VO$_3^-$, and Mg$^{2+}$. In Figure 3.8, after the addition of ADP and during a period of 10 min measurement at 520 nm, the fluorescent intensity didn’t change. Further addition of Mg$^{2+}$ in the reaction mixture increased the fluorescence at 520 nm during the time of measurement. However, the addition of ATP and Mg$^{2+}$ in the same manner did not give rise to any fluorescence change at 520 nm. Furthermore, the addition of Mg$^{2+}$ADP or Mg$^{2+}$ATP with vanadate resulted in the biggest increase in the fluorescence at 520 nm (Figure 3.9). This is consistent with vanadate inducing ADP trapping in the binding site.
Figure 3.8 Atm1p reconstituted in vesicles and structural motion monitored by fluorescent change of carboxyfluorescien in pH gradient vesicles.
3.3.10 Transport Assay

The use of Phen Green SK enclosed inside vesicles for detecting metal transport across a membrane has been described by Shingles R (84). While iron binds to the phenanthroline group in Phen Green SK, the fluorescent intensity will decrease. In this transport assay we also discovered that the addition of glutathione or EDTA will cause an increase in the fluorescent intensity of Phen Green SK, however, the reason behind this effect is still unknown. To avoid the external fluorescent signal, DPX, which is a fluorescent quencher, is always added in the reaction mixture. By the addition of iron glutathione or iron EDTA complexes, and MgATP as an energy source, the fluorescent
intensity of vesicles encapsulated with Phen Green SK decreased during the time of measurement. The fluorescence decrease provides the evidence that the iron glutathione and EDTA complexes cross the biological membrane through Atm1p (Figure 3.10a). Control experiments were always performed. The non-reconstituted liposomes with the addition of glutathione or EDTA showed no effect on fluorescence signal intensity (Figure 3.10b). Furthermore, addition of glutathione and EDTA showed a great increase in fluorescent intensity compared to the control experiments (Figure 3.10b). This also suggested that Atm1p will allow small molecules like glutathione and EDTA to pass through the channel. We also tested hepcidn-20, which is a 20 amino acid residues peptide, for iron binding, and in the transport assay. The hepcidin and hepcidin iron complex didn’t change the fluorescence intensity during the time of measurement (10 min).

Figure 3.10 Transporting Assay: (a) ferrous ion transporting assay: blue line represents a reconstituted vesicle with addition of 8 µM ferrous in the presence of 1.6 mM Mg$^{2+}$ ATP; red line represents a reconstituted vesicle with addition of 75 µM of glutathione and ferrous ion in the presence of 1.5 mM Mg$^{2+}$ ATP. (b) GSH transporting assay: blue line is a control experiment lack Atm1p in vesicles with addition of 160 µM GSH; red line represents a reconstituted vesicle with addition of 160 µM GSH in the presence of 1.5 mM Mg$^{2+}$ ATP.
3.4 DISCUSSION

The expression and purification of a membrane protein is always a tough task as a result of its hydrophobicity and toxicity to host cells, especially in *E. coli* (85). In this work, we demonstrate that we are able to over-express Atm1p with reasonable expression yield in a yeast expression system. The advantage of a yeast expression system for eukarytic membrane proteins is that the membrane protein can maintain its native structure since yeast is a eukarytic cell that can assist protein to form its correct folding. For instance, Atm1p is a mitochondrial inner membrane protein with a mitochondrial targeting pre-sequence in the gene construct in pYES2 expression vector. It is believed that the over-expressed Atm1p is finally destined to mitochondrial inner membrane with a proper folding. The over-expressed protein located in an organelle will facilitate the purification method. The strategy for purification of Atm1p is to first concentrate Atm1p by separating mitochondria from yeast cytosol and other organelles. Introduction of the His$_6$-tag at the C-terminus of Atm1p also provides a facile way for protein purification. By comparison to other membrane protein expression in yeast or other eukarytic expression systems (86), the yield of Atm1p from yeast is quite reasonable about 1 mg out of 3 g of wet yeast cells. The successful expression and purification of this ABC7 type of ABC transporter in yeast provides a novel method for the expression of other members in this family.
From the kinetics result, the $V_{\max}$ of Atm1p is faster than that of Atm1-C, which is the soluble domain of Atm1 from *Schizosaccharomyces pombe* (71). This suggests that the membrane domain of Atm1p is required for ATP hydrolysis, especially for ADP release from the ATP binding site. A conformation change in the membrane domain possibly provides a driving force for structural change of the nucleotide binding domain for ADP release. Furthermore, the Hill coefficient is 1.4, and suggests that there is cooperativity between proteins and their interaction between membrane domains is necessary for ATP hydrolysis. With a nonlinear relationship between ATPase activity and Atm1p concentration, this is also strongly consistent with our previous result for Atm1-C, which showed no cooperativity effect (71). Therefore, the membrane is truly required for this ATP hydrolysis with a higher turnover number of $1.241 \text{ min}^{-1}$.

There is still an ATPase activity for the purified Atm1p when 0.2% sodium cholate is present in the assay buffer. In general, when a membrane-bound enzyme is solubilized in detergent, either there is a low enzymatic activity or no activity with depletion of lipids. Atm1p shows a great ATPase activity in the presence of detergent in comparison with Atm1-C. The possible explanation may come from the fact that Atm1p is still associated with the endogenous lipids. However, in this case, the purification of Atm1p by Ni-NTA with an intensive wash step, it is believed that most of lipids have been removed from this protein. A finding of a reasonable ATPase activity of this purified Atm1p in the presence of detergent, it suggests that 0.2% sodium cholate still can stabilize the membrane-bound structure of Atm1p.
The specificity of Atm1p toward different nucleotides shows that the order of nucleotide hydrolytic rate is GTP>CTP>ATP>UTP. This result shows that Atm1p has a broad nucleotide specificity, similar to findings for other members of this ABC family. But the preference of Atm1p to these nucleotides is different from those of other ABC proteins. This may suggest the nucleotide specificity of this ABC transporter is not necessary for ATP hydrolysis. Especially in mitochondria, ATP is more abundant than other nucleotides, even though other nucleotides have a higher reaction rate. It is believed that ATP will be a sole substrate for Atm1p during ATP hydrolysis, when the iron sulfur is exported across the inner membrane of mitochondria.

Sodium vanadate shows an inhibitory effect with IC$_{50}$ to be 4 µM, therefore, Atm1p maybe a P-type ATPase. This is consistent to that most of ABC proteins that are P-type ATPases, however, no phosphorylation intermediate has been directly detected. In P-type ATPase, the phosphorylation of protein is an intermediate step for ATP hydrolysis (35). The mechanism of vanadate involvement in ATPase activity is to trap MgADP in the binding pocket to form a tight binding transition analogous. ADP inhibits the ATPase activity of Atm1p, because ADP is a product of this reaction. Since ADP is a product inhibitor, it is expected that the IC$_{50}$ for ADP would be much higher to be 0.9 mM. This result is also consistent with that from other members of ABC family (35).

Of various divalent metal ions for which the ATPase activity of Atm1p was measured. Mn$^{2+}$ was the best stimulator for ATP hydrolysis. Mg$^{2+}$ shows its maximal activity at 1 mM concentration, lower than Mn$^{2+}$ and Co$^{2+}$. Co$^{2+}$ is a poor stimulator for ATP hydrolysis in comparison with Mn$^{2+}$ and Mg$^{2+}$. This is also consistent with our previous study of Atm1-C and the result of HisP (35,71).
The influence of phospholipids on the ATPase activity of Atm1p shows that crude PC has an activation effect at all concentrations, and 0.1 mg/mL of cardiolipin also increases the rate of ATP hydrolysis. In the concentration 1, and 0.03 mg/mL of cardiolipin and all concentrations of PE, the ATPase activity of Atm1p is inhibited. The activation of Atm1p ATPase activity by PC may suggest that the formation of phospholipid PC-detergent micelle will stabilize the membrane structure of Atm1p, and therefore stimulate the ATPase activity of Atm1p. PE has shown that it does not form lipid bilayers, unlike PC, but forms a nonbilayer or hexagonal phase (87). This may provide the reason why there is an inhibitory effect of Atm1p in present of PE. The formation of a nonbilayer structure may lower the stability of Atm1p in this mixture, which will also result in the decrease of enzymatic activity. This also explains the activation and inhibitory effects of cardiolipin in Atm1p. The ratio between phospholipid and detergent to form a stable bilayer is important for maintaining the membrane structure of Atm1p in order to restore its ATPase activity (88).

The secondary structure determination from circular dichroism measurement shows a higher \( \alpha \)-helix content, relative to the soluble domain, and is consistent to those of membrane proteins. In most membrane proteins, the transmembrane domains contain only \( \alpha \)-helices and random loops. The higher content of these two secondary features is expected. By comparison to the secondary structure of other members in ABC family, this strongly supports the view that purified Atm1p still maintains its native secondary structure (34).
For the substrate specificity of Atm1p, the results from the transport assay suggest that Atm1p only allowed small ligands such as glutathione and EDTA passing through channel by itself or carrying iron. It also showed that Atm1p was unable to transport large peptides like hepcidin-20. This indicated that Atm1p only preferred transporting small molecular weight ligands and their metal complexes. GHS1 gene deletion in yeast indicated that cells were unable to assemble iron sulfur cluster center in cytosol (24). This result indirectly implied glutathione involved in this iron or iron sulfur cluster exporting mechanism. Further, heavy metal detoxification in vacuole from budding yeast, Ycf1 (89) or fission yeast, Hmt1 (18) has been identified as glutathione conjugate or glutathione cadmium complexes transporters. Together with our finding, we concluded that Atm1p exporting glutathione and glutathione iron complex from mitochondrion was a feasible mechanism for iron sulfur cluster assembly in cytosol. This idea was further supported by which Nfs1, a sulfur donor for iron sulfur assembly was also found existing in cytosol (90). The pre-formation of iron sulfur cluster in mitochondrion may not require for iron sulfur maturation in cytosol.

The conformational change determined by the acrylamide Trp fluorescence quenching experiment suggested ATP and ADP bound Atm1p existed in two different conformational states. With vanadate-induced ADP trapped in the active site Atm1p possesses a large structural deviation by comparison to the no substrate-bound Atm1p. The channel motion determined by proton movement in a pH gradient vesicle with entrapped fluorescien further supported ATP and ADP bound Atm1p existing in the different conformational states. When ADP is bound to the active site of Atm1p the channel is in the open conformation, whereas ATP bound to Atm1p the channel is in the
closed state. While vanadate induced nucleotide trapping in the active site has shown a great change in fluorescence intensity, this further supported the structural change determined by acrylamide Trp fluorescence quenching experiment. Together with these results, we proposed a possible mechanism (Figure 3.11) for ATP hydrolysis and substrate translocation. During ATP binding to active site of Atm1p, the channel is in its closed conformation. Upon substrate binding, it will stimulate ATP hydrolysis, phosphorylation, and subsequently drive the structural change for channel opening. After dephosphorylation, this will provide an energy source for driving substrate translocation and release. While the substrate was released, if an ATP substituted for ADP in the binding site, the channel may still maintain in its open conformation. With further substitution by ATP in the active site, the channel will return to its original closed conformation.

The analysis of conformational change and substrate specificity has provided us with a model for understanding the possible mechanism of iron or iron sulfur cluster transport and iron sulfur cluster assembly in cytosol. The further discovery of other proteins or peptides that involved in this process may provide a complete picture for iron sulfur cluster assembly in the cytosol.
Figure 3.11 Mechanism of ATP hydrolysis and Substrate Translocation.
CHAPTER 4

HEPCIDIN-20, A PUTATIVE IRON CARRIER

4.1 INTRODUCTION

Iron commonly exists in one of two oxidation states (+2 and +3). With this versatile ability of converting between ferrous and ferric ions, this makes it a very important component for redox enzymes or the electron transfer chain in the mitochondrion. However, free iron can damage cellular proteins, DNA, and membranes via generated hydroxyl radicals by reaction with hydrogen peroxide. Therefore, tight regulation of iron in the cell is necessary. The mechanism of iron homeostasis has been intensively studied for several decades. Nevertheless, the only well-established steps in iron homeostasis are iron uptake and storage. Iron trafficking and recycling inside the cell is still poorly understood.

Hepcidin or LEAP1 was first purified from human urine or plasma with antimicrobial activity (91,92). The mature peptide contains either 20 or 25 amino acid residues from the C-terminus of an 84 amino acid prepropeptide. There are eight conserved cysteine residues in its sequence, forming a β-hairpin-like structure with four intramolecular disulfide bonds (Figure 4.1) (93). The amphipathic characteristic of
hepcidin with separation of the hydrophobic and positive charge residues that disrupt the membrane potential may explain its antimicrobial activity. Unlike other antimicrobial peptides, hepcidin is well conserved throughout all organisms (Figure 4.1) (93). Hepcidin was found to predominantly express in human and mouse liver. The hepatic iron overload study by Pigeon et al. provided the first evidence that hepcidin is involved in iron metabolism in liver (94). The result indicated that iron overload induced the expression of hepcidin and iron depletion lowered its expression. During infection and inflammation, the amount of hepcidin from the infectious patient’s urine increased by 100 fold (95) and the expression of hepcidin in the infected white bass liver showed 4500 fold increase than the normal liver (96). The studies of the severe anemia of chronic disease conducted by Weinstein et al. (97) showed that the higher expression of hepcidin directly caused anemia in those patients. All these provide an evidence that hepcidin may be directly involved in iron regulation and transport.

Furthermore, from human hereditary hemochromatosis patients and HFE knockout (HFE−/−) mice model the expression of hepcidin was lower than normal (98-105). This demonstrated that the HFE gene may act as an upregulator for hepcidin expression in hepatic cells. The HFE gene has been shown to mediate iron uptake by affecting the interaction between iron-bound transferrin and the transferrin receptor. There are two other genes, which have also been identified for the regulation of hepcidin, including the transcription factor CCAAT/enhancer binding protein (C/EBPα) (106) and a macrophage cytokine interleukin-6 (Il-6) (107). Both of these proteins have been shown to stimulate the expression of hepcidin. The study of hepcidin involved in the iron regulation pathway by Frazer et al. showed that increased expression of DcytB, DMT1,
and Ireg1 correlated with lower hepcidin mRNA. When the expression of hepcidin mRNA increased, the expression of all these transporters’ mRNA always decreased. From this result, they proposed that hepcidin may act as a negative regulator for iron absorption in small intestines and iron release from macrophages. However, there is no evidence to suggest that hepcidin is directly involved in the expression or regulation of those transporters, the reason is the expression of those transporters directly controlled by IRP, which is an iron sensing regulator.

For a male adult, the total body iron is around 3000 to 4000 mg (104). The daily iron absorption is only 1 to 2 mg, which is a steady state mechanism. The overexpression of hepcidin causes iron deficiency or anemia during infection or inflammation. This didn’t explain how iron is lost by only stopping iron absorption in small intestines. Hepatic iron overload induced hepcidin expression may suggest hepcidin to act as an iron sensor. In this chapter, we provide the evidence that suggests hepcidin indeed binds to iron with a micromolar affinity.
Figure 4.1 Sequence alignment of hepcidin. The C-terminus of hepcidin from human, mouse, and rat containing 25 amino acid residues were used for sequence alignment. The alignment was performed by Multalin. The red color represents a high consensus with value greater than 90%, and the blue color represents a low consensus with value greater than 50% and lesser than 90% (93).

4.2 METHODS AND MATERIALS

4.2.1 Materials

The sequence of hepcidin-20 is “ICIFCCGCCH RSKCGMCCKT”. The custom synthetic hepcidin-20 was purchased from BIO•SYNTHESIS (Texas). Hepcidin-20 was purified by HPLC (C-18) and lyophlyzed as a white powder with MALDI-mass certificate. TCEP was obtained from Pierce Biotechnology Inc. (Rockford IL).

4.2.2 Determine Metals Binding by ESI-Mass Spectroscopy

The quantity of peptide in the oxidized form with disulfide bonds was determined by weight and its UV-vis spectrum at 280 nm with the calculated extinction coefficient
4.2.3 UV-vis Spectroscopy

A 1 mM solution of hepcidin was prepared in 50 mM Hepes, 100 mM NaCl pH7.0 buffer, and then was incubated with 10 mM TCEP at room temperature for 30 min. All buffers were degassed. The amount of hepcidin in buffer was determined by using the calculated extinction coefficient (480 M⁻¹cm⁻¹) at 280 nm. The iron binding experiment was measured at 340 nm. A 100 μM solution of hepcidin was titrated with aliquots from a ferric ion stock solution (10 or 100 mM FeCl₃ in 50 mM Hepes, 100 mM NaCl pH7.0). The final concentration of ferric ions ranged from 0 to 250 μM. The result was plotted as ΔI/I versus iron concentration. The dissociation constant was calculated based on a one site-binding model. Cobalt binding was determined by monitoring the absorption change at 310 nm, which is the charge-transfer band for cobalt thiolate. A 100 μM of hepcidin was titrated with aliquots from a cobalt ion stock solution (10 or 100 mM CoSO₄ in 50 mM Hepes, 100 mM NaCl pH7.0). The final concentration of cobalt ion ranged from 0 to 1500 μM, and the data was plotted as ΔI/I versus cobalt concentration. The dissociation
constant was calculated based on a one site-binding model (Equation 4.1), where $y$ is equal to $\Delta I/I$, and $x$ is metal ion concentration.

$$y = \frac{Bx}{K_d + x}$$  \hspace{1cm} (4.1)

4.2.4 Circular Dichroism

A 670 µM hepcidin stock solution was prepared in 50 mM Hepes, 100 mM NaCl pH 7.0. The oxidized form of hepcidin was prepared in 50 mM Hepes, 100 mM NaCl pH 7.0 to a final concentration being 100 µM. The reduced form of hepcidin was prepared in 50 mM Hepes, 100 mM NaCl pH 7.0 to a final concentration being 100 µM and treated with 1.67 mM TCEP at room temperature for 30 min. The ferrous, ferric, and cobalt bound forms of hepcidin were prepared from reduced hepcidin by adding metal ions to a final concentration of 1.33 mM. Circular dichroism spectra were measured on an Aviv model 202 circular dichroism spectrometer. Far-UV CD spectra were acquired with a 0.3-mm path-length cuvette. Spectra acquired at 20 °C were determined per 0.2 nm in triplicate and averaged. Secondary structure quantitation was determined by K2D. Buffer spectra including metal ion and TCEP were always subtracted.

4.2.5 Isothermal Titration Calorimetry.

Isothermal titration calorimetry (ITC) measurements were carried out at 25.0°C on a VP-ITC calorimeter (MicroCal, Northampton, MA). All metal and peptide stocks were prepared in the same 0.22 µm-filtered 50 mM HEPES, pH 7.0 buffer, with overnight Ar-sparge prior to use. The ferrous and cobalt stocks were prepared from Fe(NH$_4$)$_2$(SO$_4$)$_2$·7H$_2$O and CoCl$_2$·6H$_2$O, respectively, and immediately used. An
approximately 50 µM peptide sample and 1.3 mM ferrous or cobalt titrant were prepared in the HEPES buffer containing 1 mM tris(carboxyethyl)phosphine (TCEP), 5 mM dithionite, and 300 mM NaCl. A typical experiment consisted of 28 injections of 10 µL titrant into a sample with 4 min spacing to allow for adequate equilibration. Heats of dilution were determined in a separate experiment in which ferrous or cobalt was injected into the sample cell devoid of peptide and subsequently subtracted from all binding isotherms. Raw heat data was deconvoluted by integration of the heat peaks and normalized to the injection volume by use of the Origin 7 software package, and subsequently fitted to a one-site model according to the methods of Wisemen et al.

4.3 RESULTS

4.3.1 UV-vis Spectroscopy of Metal-Bound Hepcidin

The metal-bound hepcidin-20 was obtained by adding a 10 molar ratio of Fe(III) and Co(II) to the reduced hepcidin-20 in the presence of TCEP, and incubated at room temperature for 10 min. The UV-vis spectra were acquired on a Hewlett-Packard diode array spectrometer (HP 8453) with HP8453 Win system software. The $\lambda_{\text{max}}$ of Fe(III)-bound hepcidin is at 330 nm (Figure 4.2a), which is the characteristic charge transfer band from an iron thiolate complex. It is likely that Fe(III) formed a tetrahedral geometry with sulfur ligation in hepcidin. Sensitive monitory of the metal binding environment is the reason why cobalt is used as a probe for the hepcidin metal binding experiment. From the UV-vis spectrum, the absorption of Co(II)-bound hepcidin has between 310 nm and 350 nm (Figure 4.2b), which is characteristic of the ligand metal charge transfer band for
a cobalt thiolate complex. However, there is no observed absorption shown between 600-800 nm, d-d transition band for tetrahedral coordination of Co(II) (109). It is likely that Co(II) in hepcidin only coordinates to one or two sulfur groups from Cys to form an octahedral coordination, which is symmetry forbidden for d-d transitions. Therefore, it is possible that the binding environment for Fe(III) and Co(II) is different (110), and further investigations of metal binding properties maybe necessary.

4.3.2 Metal Binding Properties of Hepcidin-20 by ESI-Mass Spectroscopy

The parent peak of the ESI-mass spectrum of the reduced hepcidin-20 (Figure 4.3a) is 2199.7, which is exactly same as the theoretical mass of the reduced hepcidin-20. The Fe(II) and Fe(III) bound hepcidin spectrums (Figure 4.3b) only showed one iron attached to hepcidin-20. The Co(II)-bound hepcidin-20 mass spectrum (Figure 3c) indicates that at least two Co(II) ions are bound to this peptide. However, the number of metals bound to hepcidin determined from mass spectroscopy may not represent the actual binding properties of the peptide. Since the mass determination was performed in a gas phase, and weak binding may not be detected in this condition. But at least it provides the evidence of metal ions bound to the peptide.
Figure 4.2. UV-vis Spectroscopy of metal-bound hepcidin. (a) UV-vis spectrum of ferric ion bound hepcidin, $\lambda_{\text{max}}$ at 330 nm is the charge transfer band from an iron thiolate complex. (b) UV-vis spectrum of cobalt ion bound hepcidin, $\lambda_{\text{max}}$ at 310 nm is the charge transfer band from a cobalt thiolate complex.
Figure 4.3 ESI-mass spectroscopy of reduced hepcidin and hepcidin metal complexes. (a) Reduced hepcidin. The measured molecular weight of reduced hepcidin is 2199.7. (b) Iron-bound hepcidin. The molecular weight of one iron-hepcidin complex is 2254.7. (c) Cobalt-bound hepcidin. The molecular weight of one-cobalt and two-cobalt bound hepcidin complexes are 2256.6 and 2313.5, respectively. Cont.
Figure 4.3 Cont.

Reduced Hepcidin

+Fe

Cont.
Reduced Hepcidin

+Co  +2Co
4.3.3 Determination of Metal Dissociation Constant by UV-vis and Isothermal titration calorimetry (ITC)

The sulfur to metal charge transfer band provides a good indicator for probing metal-ligand binding properties, because of the higher extinction coefficient compared to other ligands. The Fe(III)-hepcidin dissociation constant was measured by following the wavelength at 340 nm. Fe(III) binding (Figure 4.4a) is plotted as $\Delta I/I$ versus iron concentration. The $K_d$ for Fe(III) was fitted based on a one-site binding model with equation as $y = Bx/(K_d+x)$, where $\Delta I/I$ is a fraction of bound form versus total peptide, $x$ is the concentration of metal, and in this equation, $B$ is set to $B_{\text{max}} = 1$. The $K_d$ of Fe(III) binding is 12 $\mu$M. Co(II) binding (Figure 4.4b) is also plotted as $\Delta I/I$ versus cobalt concentration and fitted by a one-site binding model as previously described Equation 4.1. The $K_d$ of cobalt binding is around 60 $\mu$M. Iron binding was further investigated by ITC (Figure 4.5), which not only provides the binding constant but also shows the enthalpy change of the binding reaction. The $K_d$ of Fe(II)-hepcidin binding is 6 $\mu$M, which is comparable to that determined from UV-vis experiments. The $\Delta H$ for Fe(II) binding to hepcidin-20 is -778.3 cal/mole. The discrepancy between these two measurements may due to the lower pH of Fe(III) stock solution which diminished the binding affinity.
Figure 4.4 Metal binding constants determined by UV-vis spectroscopy. (a) Iron binding. (upper) UV-vis spectra of a ferric-hepcidin complex with increasing ferric ion concentration. (lower) Ferric hepcidin binding plot. The y axis is the ratio of absorbance change at 340 nm. x axis is the concentration of ferric ion. (b) Cobalt binding. UV-vis spectra of the cobalt-hepcidin complex with increasing cobalt ion concentration. (lower) Ferric hepcidin binding plot. The y axis is the ratio of absorbance change at 310 nm. The x axis is the concentration of cobalt ion. Cont.
Figure 4.4 Cont.

(b)
Figure 4.5. Determination of iron binding by ITC. (upper) The raw heat response of hepcidin ferrous ion titration. (lower) The binding plot of hepcidin and ferrous ion.
4.3.4 Circular Dichroism

The secondary structure of the oxidized, reduced and metal-bound hepcidin-20 was determined by circular dichroism (Figure 4.6). The composition of the secondary structure was calculated by K2D (78,79), which is a web-based program for secondary structure prediction from CD spectrum ranging from 200 to 241 nm. The predicted secondary structure of the different forms is summarized on Table 4.1. From the CD spectrum, it is clear that the secondary structure of oxidized hepcidin is similar to that determined from the NMR study. After reduction by TCEP, the secondary structure of hepcidin-20 is completely transformed to $\alpha$-helix. This suggested that the disulfide bonds from the oxidized form have been totally broken. Addition of Co(II) into the reduced hepcidin only slightly perturbs the secondary structure, whereas follows addition of Fe(III) and Fe(II) into the reduced hepcidin, the secondary structure returned to that of the oxidized form—containing both $\beta$-sheets and random coil and comprising four disulfide bonds. This dramatic structural change may imply that iron must play an important role in maintaining the full function of the peptide, either for signal transduction or iron recycling.
Figure 4.6. Circular Dichroism of hepcidin and hepcidin metal complexes. --- is oxidized hepcidin. --- is reduced hepcidin. … is cobalt-hepcidin complex. —— is ferrous-hepcidin complex. —— is ferric-hepcidin complex.
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</table>

Table 4.1 Secondary structure of hepcidin and hepcidin metal complexes determined by CD. The percentage of each secondary structure was determined by K2D. The percentage of the secondary structure of oxidized hepcidin from NMR was estimated from the amino acid sequence.

4.4 DISCUSSION

Hepcidin was first isolated from human urine and plasma and showed significant antimicrobial activity. Together with the different model studies in vivo, hepcidin has been suggested to serve as a negative mediator for iron absorption in small intestines and iron release from macrophages. With eight conserved Cys in the sequence, it is a characteristic for signal hormone. Patients carrying infection showed a great increase in the amount of hepcidin in urine, and was typically increased around 100 fold increasing compared to normal people (95). Also the overexpression of hepcidin can cause inflammation anemia. However, iron uptake is a steady state mechanism, this means the iron absorption only 1-2 mg per day for a male adult (104). Combined with all these results, it didn’t explain how this signal peptide would cause the iron deficiency during the infection via only stopping iron absorption. The iron binding properties of hepcidin
have not yet been investigated, and so it was of interest to determine its iron binding of this peptide may play an important role in iron regulation, especially for infection anemia.

In this chapter, we first investigate the metal binding properties of hepcidin-20. From the iron binding experiment of hepcidin, it is suggested that hepcidin may act as an iron carrier for iron recycling or removal. The overexpression of hepcidin in mice model with iron deficiency may implicate the possible function and mechanism for iron recycling and removing (95). Antimicrobial activity of hepcidin has been observed for following high expression during infection. The iron binding ability with the moderate binding affinity helps to explain the ability to kill bacterial by iron depletion. Since most bacteria have evolved a function for acquiring iron even in iron deficient environment. From the CD spectrum, the reduced hepcidin adopts only $\alpha$-helix in its secondary structure with hydrophobic in N-terminus and positive charges in C-terminus. With this separation of hydrophobic and positive charge in a $\alpha$-helix structure, it is a characteristic of an antibiotic by forming a channel in bacterial membranes. The formation of a voltage-dependent channel in bacteria membrane, which will destroy a cell’s transmembrane potential, may also explain the antimicrobial activity of hepcidin.

The metal binding properties of hepcidin display the different preference between iron and cobalt. By adding ferric or ferrous ions into a reduced peptide, the dramatic structural rearrangement from $\alpha$-helix to $\beta$-sheet and random coil (Figure 4.7) might indicate that iron is required for the full function of hepcidin in iron homeostasis as a signal peptide and an antimicrobial activity during infection and inflammation.
Figure 4.7 Model of hepcidin conformational change between reduced form and metal bound form, with addition of iron and reducing agent. Under reducing conditions, the secondary structure of hepcidin contains only α-helix. With binding to iron, the secondary structure of iron-hepcidin complex is similar to the secondary structure of the oxidized hepcidin.
CHAPTER 5

FUNCTIONAL ROLE OF A CONSERVED TRYPTOPHAN RESIDUE OF CHROMATIUM VINOSUM HIGH POTENTIAL IRON PROTEIN

5.1 INTRODUCTION

The iron–sulfur cluster is a metalloprosthetic center that demonstrates a wide variety of biological functions (111,112). An important, and as yet unresolved problem is the characterization and understanding of the electronic and structural factors that define the chemistry of the cluster in diverse biological contexts (113,114). A property that is now recognized to be of functional relevance is cluster stability, and in recent reports we have both identified pathways for cluster degradation, and evaluated the factors that contribute to cluster stability (115-117). These studies have been carried out on the high potential iron protein from Chromatium vinosum, a structurally well-defined iron–sulfur cluster protein that has been cloned and overexpressed (118), and affords a useful paradigm for structure–function studies. We (115,119-121), and others (122), have previously found that Tyr19, Phe48, and Phe66 (C. vinosum numbering) maintain the hydrophobic pocket around the iron-sulfur cluster and inhibit hydrolytic degradation of the 4Fe-4S prosthetic...
center in the oxidized state. In those cases where partial destabilization of the cluster is achieved (for Phe48 and Phe66 mutants) a \([\text{Fe}_3\text{S}_4]\) degradation intermediate was identified (120). Studies of point mutants at these sites have provided useful insight on the factors that control the redox and electron-transfer properties of the cluster (115).

Here we focus attention on the conserved residue Trp80. This also contributes to the hydrophobic character of the cluster binding pocket and has been invoked as a mediator of electron transfer. The role of this residue has been investigated by isolation and characterization of a Trp80Asn mutant where the polar Asn side chain replaces the conserved indole ring. The influence of the non-conservative polar center on the reduction potential of the cluster, and the effect on solvent accessibility and cluster stability, are assessed. Also, the temperature-dependent stability of the Trp80Asn mutant is compared with those of Tyr19Leu, Phe66Ser and Phe66Asn mutants, and with native protein. In contrast to the Tyr and Phe aromatics, Trp80 demonstrates a distinct role in defining protein stability.

5.2. METHODS AND MATERIALS

5.2.1. Mutagenesis and Protein Isolation

Protocols for site-directed mutagenesis and characterization of mutant clones have been described elsewhere (115). Cell cultures were grown, expression induced by IPTG, and initial protein purification was carried out as previously described (118). Final purification was carried out by FPLC (Pharmacia) on a Mono Q column (1 × 5 cm) using non-denaturing conditions. Optimal separation of bands was obtained by a gradient
method, using two stocks of degassed phosphate buffer, at pH 7.5 (A: 10 mM phosphate and B: 10 mM phosphate, 500 mM NaCl). The total running time was 20 min (3 min with 0% B, and 17 min from 0 to 100% B). Protein concentration was estimated by use of the published extinction coefficient for the cluster absorption at 388 nm ($\varepsilon_{388} \sim 16.1$ mM$^{-1}$ cm$^{-1}$) for the reduced native protein (123), with the assumption that this would be similar for the Trp80Asn mutant.

5.2.2. Electronic Absorption and Electrochemical Methods

Optical spectra were obtained at ambient temperature by use of a Hewlett-Packard 8452A diode array spectrophotometer. Reduction potentials for recombinant native and mutant HiPIP were determined by spectrochemical titration methods (124). A 1 mL of 20 $\mu$M native or mutant HiPIP in 10 mM NaHPO$_4$, 100 mM NaCl pH 7.0 at RT was titrated with a 2 $\mu$L of potassium ferrocyanide from a 50 mM stock solution in 10 mM NaHPO$_4$, 100 mM NaCl pH 7.0. The absorbance change at 500 nm was measured to determine the mid-point reduction potential ($E_M$).

5.2.3. NMR Spectroscopy

1D $^1$H NMR spectra were acquired at 600 MHz on a Bruker spectrometer at 300 K. Recombinant native and mutant HiPIP NMR samples were concentrated and equilibrated in 50 mM potassium phosphate buffer, pH 6, by Amicon ultrafiltration. The concentrated sample was then lyophilized and dissolved in D$_2$O. The resulting sample solution contained approximately 2-3 mM protein. Oxidized samples were prepared by addition of a crystal of potassium ferricyanide. All one-dimensional spectra were
acquired using the SUWEFT(180-τ-90 AQ) pulse sequence in order to optimize resolution in the paramagnetic region with retention of very effective solvent suppression (125). Samples were prepared in H$_2$O and contained 10% (v/v) D$_2$O for the lock.

5.2.4. EPR

EPR spectra were recorded at X-band with a Bruker ESP 300 spectrometer equipped with an Oxford liquid helium cryostat and an internal integration program in the Bruker software package. The temperature was measured by use of an Au/Fe versus Cr thermocouple. Experimental parameters are listed in footnotes to the tables and figure legends. Typical spectral conditions: microwave frequency = 9.46 GHz; power = 1 mW; modulation frequency = 100 kHz; modulation amplitude = $10^{-3}$ T.

5.2.5. Evaluation of Hydrolytic Stability

Kinetic data for cluster degradation were obtained at an ambient temperature of 295 K by monitoring the decrease in absorbance at 500 nm for reduced and oxidized mutant proteins by use of a Hewlett-Packard 8452A diode array spectrophotometer. Typical experimental conditions included a solution of 20 µM HiPIP in 10 mM sodium phosphate buffer with 0.1 M NaCl (pH 6.0). Approximately 100 µM oxidant was added from a concentrated stock solution and the assay started immediately after mixing by inversion. The result of the assay (OD decay at 500 nm) was then fitted to a single-exponential curve and the apparent rate constant obtained from the fitting (120).
5.2.6. Evaluation of Thermal Stability for Native and Mutant HiPIPs

The thermal stability of both the mutant and native HiPIPs was monitored by use of a Hewlett-Packard 8452A diode array spectrophotometer. Optical spectra were obtained every 5 °C with increasing temperature under anaerobic condition at 388 nm. The melting temperatures ($T_M$) for the mutant and native HiPIPs were determined from the break point in plots of absorbance versus temperature (Fig. 5.1).

5.2.7. Electron Transfer Rates for Mutant and Native HiPIPs

Kinetic data were collected on an OLIS RSM-100 stopped-flow UV-Vis spectrophotometer with a 20 mm cell path length. Time-dependent absorbance changes were monitored at 500 nm over 4 s. Recombinant native and mutant HiPIPs were equilibrated in 0.1 M phosphate buffer, pH 7.0, by Amicon ultrafiltration to give a sample solution of approximately 0.1 mM. Oxidant solutions of 1 mM $K_3Fe(CN)_6$ were prepared in 0.1 M phosphate buffer, pH 7.
5.3. RESULTS

5.3.1. Characterization of the Trp80Asn Mutant

The mutant (Trp80Asn) was characterized and expressed in *Escherichia coli* as holoprotein and isolated in the reduced form following standard protocols. The expression yield for the mutant was similar to that obtained for recombinant native protein (~ 30 mg l⁻¹). Optical spectra for reduced and oxidized native and mutant proteins (Figure 5.2), respectively, were found to be similar, with a small decrease observed in the 282 nm absorbance, as expected following loss of a Trp residue. Our assumption that the concentration of the mutant protein can be assessed from the cluster extinction coefficient for the native protein at 388 nm appears justified by the fact that calculation of \( \varepsilon_{282} \) (M⁻¹
For Trp80Asn by subtracting the contribution for Trp $\varepsilon_{282} = 5500 \text{ M}^{-1} \text{cm}^{-1}$) from the native extinction at 282 nm gives an expected value of $35.8 \text{ M}^{-1} \text{cm}^{-1}$, in good agreement with the experimental value of $36.1 \text{ M}^{-1} \text{cm}^{-1}$.

The EPR spectrum of the oxidized Trp80Asn mutant (Figure 5.3), measured immediately after ferricyanide oxidation, was found to be very similar to that obtained for recombinant native HiPIP (Table 5.1), although the prominent signals from the oxidized center were observed to be diminished, depending on the delay time before freezing the oxidized sample as a result of the degradation reaction. No evidence for transient formation of a $[\text{Fe}_3\text{S}_4]^+$ cluster signal was found, such as was observed during decay of oxidized Phe66 mutants.

$^1$H chemical shifts (Figure 5.4) for paramagnetically-shifted signals in the reduced mutant protein are summarized in Table 5.2. Resonances from hyperfine-shifted protons in the reduced mutant show no significant difference from those of the native spectrum. The NMR spectrum of the oxidized mutant could not be measured since the degradation rate and autoreduction pathways occurred rapidly at the concentration required for the NMR experiment.
Fig. 5.2. Comparison of optical spectra for reduced native HiPIP and the Trp80Asn mutant. Solutions were prepared such that the cluster absorbance at $A_{388}$ (and therefore solution concentration) was similar for each. The native spectrum is shown by the dashed line.
Figure 5.3. EPR spectrum of mutant W80N HiPIP.
<table>
<thead>
<tr>
<th>$g$ values</th>
<th>Rec-native</th>
<th>Trp80Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g^\perp$</td>
<td>2.122</td>
<td>2.119</td>
</tr>
<tr>
<td>$g^\parallel$</td>
<td>2.040</td>
<td>2.037</td>
</tr>
</tbody>
</table>

Table 5.1. EPR parameters for oxidized native and Trp80 mutant

<table>
<thead>
<tr>
<th>Assignment$^a$</th>
<th>Rec-native</th>
<th>Trp80Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>C43$\beta$</td>
<td>17.02</td>
<td>17.34</td>
</tr>
<tr>
<td>C46$\beta$</td>
<td>11.81</td>
<td>11.10</td>
</tr>
<tr>
<td>C63$\beta$</td>
<td>15.90</td>
<td>15.95</td>
</tr>
<tr>
<td>C77$\beta$</td>
<td>12.95</td>
<td>13.10</td>
</tr>
</tbody>
</table>

Table 5.2. $^1$H NMR shifts obtained at 300 K for the isotropically-shifted resonances of reduced native and mutant HiPIP. $^a$ Assignments are from Refs (114,126).
Figure 5.4. NMR spectrum of mutant W80N HiPIP.
5.3.2. Evaluation of Mutant Stability - Hydrolytic and Thermal

In comparison with the polar mutants of Tyr19, the Trp80Asn mutant was found to be relatively stable in the reduced state, at ambient temperature and pH 7.0, and could be maintained for many weeks as frozen stocks (-20°C). In the oxidized state the mutant protein was generally unstable over a time-frame that was typically on the order of 30 min to several hours, depending on the solution temperature, with greater stability observed at lower temperature. Following addition of a fivefold excess of potassium ferricyanide, the mutant protein formed a bright-green solution, presumably due to formation of Prussian blue \( [K^+ + Fe^{3+} + Fe(CN)_{6}^{4-} \rightarrow (KFe(CN)_6Fe)_x ] \) after oxidation and degradation of the cluster. After rapid removal of excess oxidant by passage through a short G-25 column

\[
(0.8 \times 2.5 \text{ cm})
\]

the optical spectrum revealed the absence of the cluster; however, incubation with DTT- FeCl\(_2\)-Na\(_2\)S for 40 min led to reconstruction of holoprotein, which was further characterized following gel filtration chromatography.

The experimentally determined melting temperatures obtained from the temperature dependence of cluster absorbance at 388 nm for Trp80Asn, Tyr19Leu, Phe66Ser, Phe66Asn, and native are summarized in Table 5.3. Native HiPIP shows a considerably higher \( T_M \) (±81°C) relative to any mutant, and the \( T_M \) for Trp80Asn is considerably lower than other mutants that show greater solvent accessibility.
Table 5.3. Thermal stabilities of native and mutant HiPIPs. $^a T_M$ is defined as shown in Fig. 5.1.
5.3.3. Reduction Potential and Electron Transfer Characteristics of the Trp80Asn Mutant

Reduction potentials were estimated by standard spectrochemical titration methods. The experimentally determined mid-point reduction potential ($E_M$) obtained at 298 K for the Trp80Asn mutant HiPIP (395 mV) was shifted by $+33$ mV relative to the potential for recombinant native (362 mV). This is consistent with the replacement of a neutral for a polar residue, however, the effect is minor and of no significance since we have previously observed larger increases in Tyr19 mutants where neutral non-polar side-chains have replaced the aromatic ring (115).

Absorbance changes show that one electron of reduced HiPIPs is transferred in solution to Fe(CN)$_6^{3-}$. The color changes of oxidation of native and mutant Trp80Asn HiPIPs have been previously described. The kinetic data was collected at 500 nm within 4 s (Figure 5.5) and was readily fit by a first order exponential decay function. The electron transfer rate constants for native and mutant Trp80Asn HiPIPs are 0.7 and 1.7 s$^{-1}$, respectively. While Trp80 has been invoked to play a role in mediating electron transfer in HiPIPs through the $\pi$-electron clouds of the aromatic core, the electron-transfer rate for mutant Trp80Asn only differs by a factor of 2.4, and is readily explained by the change in midpoint potential of the cluster that increases the driving force. This observation is consistent with our previous observations of electron-exchange reactions of mutants, and with the pathway approach of Beratan et al., which tends to emphasize $\sigma$-bonding pathways in mediating electron transfer through a protein framework (127).
Again, our experimental data suggest that the role of the aromatic core residues is to regulate solvent access and maintain structural stability rather than to mediate cluster redox potentials or electron transfer.
Figure 5.5. Kinetic traces of electron transfer of native(right) and mutant W80N(left) HiPIPs by stop-flow.
5.4. DISCUSSION

A non-conservative mutation has been made at residue Trp80 of *C. vinosum* HiPIP. The influence on the cluster reduction potential of this mutant was found to be consistent with the Phe66Asn mutant with a modest increase of $E_M$ relative to the native protein and favorable electrostatic stabilization of the reduced cluster (120). The perturbations observed for the shifted resonances in the $^1$H NMR spectrum of the Trp80Asn mutant (Table 5.2) were minor and indicate the absence of substantial perturbation of the electronic structure of the cluster. In the oxidized state, the EPR signature was also found to be similar to the native signal.

Previously we have demonstrated by a rational mutagenesis approach, that none of the obvious parameters (solvent accessibility, electrostatic charge effects, H-bonding, π-contacts) can be used to explain the large range of $E_M$s that differentiate protein-bound high and low potential Fe-S centers (a 1 V range) (115-121). Rather, our results support a modest dipolar influence from the amide substitution for a core aromatic residue (119). The extent of solvent accessibility was found to be significantly lower than that exhibited by Tyr19 mutants (115), consistent with the relative locations of these two residues. Tyr19 is located at the entrance to the hydrophobic cluster binding pocket and maintains a hydrophobic barrier for exclusion of water from the cluster cavity, while Trp80 lies within the pocket — although one edge of the side-chain appears to be surface accessible. Degradation of the cluster arises only for the oxidized center and appears to result from cluster hydrolysis, consistent with the solvent accessibility patterns observed for the mutant proteins reported from our laboratory.
Although the solvent accessibility to the cluster pocket is diminished for Trp80 mutant, relative to those for Tyr19 or Phe66, the data from the temperature-dependent stability assay clearly shows that Trp80Asn has the lowest thermal stability, compared with Tyr19, Phe48 and Phe66 mutants. Previously the relative stabilities of native and Tyr19 mutant HiPIPs have been quantitatively evaluated by differential scanning calorimetry (122). A three-step model that included the reversible endothermic unfolding of the polypeptide (step 1), the exothermic release of the 4Fe-4S cluster (step 2), and an irreversible transition to the final state at high temperatures (step 3) has been proposed (122). Assuming the release of the cluster to be a constant factor, the low \( T_M \) obtained for the Trp80Asn mutant in the thermal stability assay suggests that this residue plays an important role in defining the reversible endothermic unfolding of the peptide in step 1. These data again demonstrate a quite distinct role from what has been previously postulated for the aromatic core residues. In the oxidized state the cluster is sensitive toward hydrolytic decomposition. The hydrophobic aromatic core residues apparently maintain a barrier toward exclusion of solvent. Results from Tyr19, Phe48, and Trp80 mutants indicate that such residues have little role in defining the reduction potential of the cluster. Apparently the hydrophobic aromatic residues in the cluster core form a barrier to solvent accessibility. In the case of Trp80, solvent access must be mediated by conformational flexibility of the backbone that is introduced following the non-conservative mutation.
6.1 Introductions

RNA as a drug target has been arouse the scientists’ interesting in discovery of new antibiotics, since there were many antibiotic resistant strains appearing at an increasing rate (128). The reasons for RNA as a potential drug target are the diversity and complexity of RNA structures including secondary and tertiary structures that provide for the protein recognition and small molecular targeting (128,129). First, RNA and binding proteins play an important role in storage of genetic information, formation of genetic materials, and enzyme activity. Therefore, the disruption of the interaction between RNA and its binding protein would provide as a potential therapeutic way against bacterial infections. Second, unlike DNA, single strain RNA can form complicated tertiary structure due to base mismatch such as bulges, loops, stems, pseudoknots, and hairpins. Because of these three dimensionally structural motifs, RNA binding proteins or small molecules would recognize these features due to structural accessibility, not tight pack as DNA. Also the recognition of the duplex DNA with binding-proteins is based on the
sequence of DNA, but for RNA the recognition is depended on the structure, not on sequence. Third, the lack of cellular repair system of RNA is also another attractive reason for drug targeting. However, there are several RNA tumor viruses and other retroviruses containing single strains of RNA that are folded into a variety of secondary and tertiary conformations, these will provide those RNA molecules as potential drug targets (130-136).

Aminoglycoside antibiotics that selectively disrupt RNA-protein interactions have shown efficiently therapeutic value against Gram-positive and Gram-negative bacteria and mycobacterial infections (137,138). These molecules, however, have been demonstrated to inhibit protein translation by recognition of 16S rRNA (139,140), to inhibit the binding of the HIV Rev protein to its viral RNA recognition element, the Rev response element (RRE)(141), and also to inhibit the activity of hammerhead ribozymes (142), group I intron ribozymes (143), and the binding of HIV Tat peptide to Tat-RNA (144). All these binding interaction in vitro have studied detail by several techniques such as NMR (145), X-ray crystallography (146), surface plasmon resonance (147), biosensor (147,148), and other thermodynamic methods(131). More recently we have demonstrated the thermodynamic parameters for RNA and aminoglycoside complex formation and the principal mechanism for recognition and binding of aminoglycoside and RNA by isothermal titration calorimetry (131). Also aminoglycosides contain several amino groups with highly positive charges at neutral pH and hydroxyl groups in saccharide ring A and B, therefore, electrostatic effect and hydrogen bonding provide an essential role in
recognition and binding affinity of those RNAs. These detailed studies of specific recognition and binding modes provide us much more knowledge to develop new potential antibiotics in future.

From the aminoglycosides and RNA binding studies, we realize that most aminoglycosides only can form tightly bindings with target RNAs and disrupt protein and RNA interaction, but not damage the binding RNAs. Recently the efforts on the development of metal derivatives of aminoglycosides have shown very impressive progresses. In our group, we have demonstrated that copper derivatives of aminoglycosides (Figure 6.1) (149) degrade cognate RNA targets at concentrations as low as picomolar levels at physiological pH and temperature with highly specific cleavage of RNA targets and also mediate catalytic cleavage of DNA \textit{in vitro}. These metal derivatives of aminoglycosides show that they not only recognize the specific binding sites but also cleave their target RNAs (130,132,149-151). However, all these binding properties and structures between RNAs and binding proteins or small molecules, and cleavage chemistry of RNAs with metal derivatives of aminoglycosides have been only done \textit{in vitro}, rare \textit{in vivo} assays have been published so far, especially cleavage chemistry of metal aminoglycosides. Recently Werstuck G. and Green M. R. (152) have demonstrated that small molecules aptamers bound kanamycin A and tobramycin \textit{in vivo} with introduction of these aptamers into expression vectors and testing them for drug-resistant phenotype in \textit{Escherichia coli}. They also showed the ability of small molecules, Hoechst dye 33258 and 33342, to repress translation of specific genes with insertion of aptamers into the 5’ untranslated region of messenger RNAs in mammalian cells.
Bioluminescent systems, such as Luciferase, β-Galatosidase and Green Fluorescent Protein (GFP) (137,138,153-155), have been exploited as molecular probes for analysis of gene expression and cell biology markers in different organisms. For luciferase and β-galatosidase activity assay, they are required exogenously added substrates or co-factors. Unlike these two systems, GFP of the jellyfish *Aequorea Victoria* has been shown to emit green fluorescence with emission maximum at 509 nm when it was irradiated with light of the appropriate wavelength with excitation wavelength at 395 and 470 nm. The active chromophore of GFP is generated via the spontaneous cyclization and oxidation of a serine-dehydrotyrosine-glycine trimer within a defined hexapeptide sequence of the protein (153). In this chapter, we utilize the advantages of GFP as reporter gene for *Escherichia coli* with introduction of deoxyribonucleotide complementary sequences of 23-mer aptamers (R23) in 3’-end prior the stop codons. When this R23 sequences in mRNA was recognized and cleaved *in vivo*, the fluorescent intensity of GFP would decrease due to the destruction of mRNA of GFP (Figure 6.2) (156,157). Therefore, it would provide as a powerful probe for the screening of novel drugs in cleavage of pathogenic mRNA sequences, and also can be used as a tool for combinatorial chemistry.
Figure 6.1. Copper kanamycin A (left) and an experimentally determined structure (right) of aminoglycoside bound to target R23 RNA.
Figure 6.2. Schematic illustration of GFP mRNA transcripts. The upper sequence is translated to GFP with retention of fluorescence. The lower sequence is cleaved before the stop signal and translation of the polypeptide chain is aborted.
6.2. METHODS AND MATERIALS

6.2.1 Construction of GFP-C2 into pET-21b(+) (pET-21b-GFP-C2)

The gene for GFP protein was taken from the commercial vector pGFP-C2 (Clontech) by restriction digestion and cloned into the NheI/EcoRI digested pET-21b(+) expression vector (Novagen). The sequence of expression vector pET-21b-GFP-C2 was finally confirmed by DNA sequence analysis and the resulting plasmid (pET-21b-GFP-C2) was subsequently transformed into *Escherichia coli* strain BL21(DE3) (158).

6.2.2 Construction of pGFP-C2-R23

The complementary DNA sequences of R23 (5’-AGC TTG GCC TGG GCG AGA AGT TTA GGC CG-3’ and 5’- GAT CGG GCC TAA ACT TCT CGC CCA GGC CA-3’) was introduced into the HindIII/BamHI pGFP-C2 digested vector.

6.2.3 Construction of GFP-C2-R23 into pET-21b(pET-21b-GFP-C2-R23)

Subsequently the gene for the GFP-R23 fusion protein was obtained from pGFP-C2-R23 by restriction digestion and cloning into the NheI/BamHI sites of pET -21b(+). The sequence of expression vector pET-21b-GFP-C2-R23 was finally confirmed by DNA sequence analysis and the constructed plasmid (pET-21b-GFP-C2-R23) was subsequently transformed into *Escherichia coli* strain BL21(DE3) (158).
6.2.4 Expression of pET-21b-GFP-C2 and pET-21b-GFP-C2-R23 in BL21(DE3)

Both pET-21b(+)-GFP-C2 and pET-21b(+)-GFP-C2-R23 plasmids were expressed in *Escherichia coli* strain BL21(DE3) with ampicillin selection. Cells were grown to an OD$_{600}$ of 0.8±0.1, and overexpression was induced with 1mM IPTG at 37°C incubation. After 4 hours expression the cells were harvested and washed once with PBS buffer.

6.2.5 Fluorescent Measurement of pET-21b(+)-GFP-C2 and pET-21b(+)-GFP-C2-R23 Proteins

The cells pellets from the pET-21b(+)-GFP-C2 and pET-21b(+)-GFP-C2-R23 plasmid cell lines were resuspended in 400µL PBS and the fluorescence measured by fluorimetry with excitation wavelength 395nm and emission wavelength 505nm for the whole cells’ fluorescent assay. The resulting spectrum is shown in Figure 6.1. Alternatively, the cells pellet of the pET-21b(+)-GFP-C2 cell line was lysed for 30 min with 1% triton and lysozyme in PBS buffer on ice with vigorous vortexing. After centrifugation (>14000rpm), the supernatant was measured by fluorometer with the prior condition.

6.2.6 Copper Kanamycin Cleavage of R23 *in vivo* Assay by Fluorescent 96-well Reader

Cells were grown to an OD$_{600}$ of 0.8±0.1 in 100 mL LB, and IPTG and ampicillin were added to final concentration of 1 mM and 50 µg/mL, respectively. The resulting cell culture was divided into 5 mL aliquots in 50 mL Corning tubes of copper kanamycin
or kanamycin (control experiment) added to a final concentration of 0, 1, 2, 4, 6, 10, 20, 40, and 80 µM. These were incubated at 37 °C for 4 hours, and the cells were harvested and washed 1x with PBS buffer. Finally, the cell mass was lysed with 1% triton and lysozyme in PBS buffer for 30 minutes on ice with vigorously vortex. The fluorescence readings for each reaction were collected from a fluorescent 96-well reader. The data from the cleavage assay was plotted as the fluorescent intensity against final concentration of copper kanamycin or kanamycin in cell culture.

6.3 RESULTS

6.3.1 Construction of gfp and gfp-r23 Genes into Expression Vector pET-21b(+)

The gfp gene from pGFP-C2 plasmid was cloned into E. coli expression vector pET21-b(+) to test whether this gene could be expressed in bacteria and determined of that the expressed GFP protein was functional in this organism. The gfp gene with a 771bp fragment from pGFP-C2 between Nhe1 and EcoR1 was inserted into pET-21b(+) and the resulting pasmid was termed as pET-21b-GFP-C2. However, the complementary DNA sequences of R23 were first introduced into pGFP-C2 vector, and the gfp-r23 fragment was cloned as a NheI-BamHI insert into pET-21b(+) as a result of pET-21b-GFP-C2-R23. These two resulting plasmids were first amplified in DH5α and finally transformed into a BL21(DE3) expression system (158).
6.3.2 Expression of GFP in *Escherichia coli* and Spectrofluorimetric Detection

When pET-21b-GFP-C2 and pET-21b-GFP-C2-R23 were expressed in BL21(DE3), the expression efficiency was detected via the emission maximum at 509 nm. The optimal condition for GFP overexpression was four hours after IPTG induction, which shows the highest emission intensity at 509 nm. The fluorescent spectra (Figure 6.3) of GFP that were characterized under whole cells condition or cell extract with the excitation wavelength at 395 nm and emission wavelength at 509 nm show similar spectral properties to the previous reports. Further, fluorescence characterization of the GFP-R23 fusion protein (Figure 6.3) from cell extracts has shown similar spectral properties to GFP itself in *E. coli*.

![Fluorescence spectra](image)

Figure 6.3 Fluorescence spectra of cells harboring plasmid with normal GFP (blue), with R23-tagged GFP (red), and from cell extracts containing normal GFP (green). Data was obtained from similar numbers of cells grown under similar conditions. Fluorescence spectra were obtained with $\lambda_{\text{ex}} = 395$ nm. The characteristic GFP emission with $\lambda_{\text{em}} \sim 509$ nm was observed.
6.3.3 Monitoring *in vivo* R23 Apatmer Cleavage with Copper Kanamycin by use of a 96-well Fluorescent Reader

To test whether the R23 sequence had been cleaved by copper kanamycin *in vivo*, we utilized the advantage of a spectrofluorescent 96-well reader that would provide high-throughput screening. The cells were grown to log phase and induced with IPTG, and cleaved *in vivo* with a concentration of copper kanamycin ranging from 1 to 80 µM. The fluorescent emission readings at 509 nm of each reaction were collected by use of a 96-well reader and excited at 395 nm. The results of copper kanamycin cleavage of GFP and GFP-R23 were plotted as fluorescent intensity versus final concentration of copper kanamycin in cell culture, shown as Figure 6.4. The results showed that the expression of pET-21b-GFP-C2 wasn’t affected with incubation of copper kanamycin, however, the expression of pET-21b-GFP-C2-R23 was shown to result in a decrease in the fluorescence intensity with increasing concentration of copper kanamycin. This supported cleavage of R23 by copper kanamycin *in vivo*. Also the control experiments of GFP and GFP-R23 with incubation of kanamycin have shown no fluorescent intensity change over a similar range of concentration of kanamycin, as shown in Figure 6.4. This eliminates the possible inhibition of expression by kanamycin itself.
Figure 6.4. Intracellular cleavage of GFP-R23 mRNA transcripts by the copper complex of kanamycin A (black) resulting in a decrease in GFP production and fluorescence intensity. The difference in fluorescence intensity (F.I. difference), relative to background, is plotted against the concentration of copper kanamycin A added to the culture medium. Control assay with kanamycin A (red).
6.4 DISCUSSION

Previously we have demonstrated copper derivatives of aminoglycosides (Fig. 6.1) to mediate highly specific cleavage of cognate RNA targets at concentrations as low as picomolar levels at physiological pH and temperature. Structural and thermodynamic analyses of the aminoglycoside–RNA complex, and the cleavage chemistry of the complex under hydrolytic and oxidative conditions have been assessed. However, demonstration of the efficacy of such reagents in vitro is only a first step. To demonstrate in vivo cleavage chemistry we have designed a fluorescence assay based on use of the green fluorescent protein (GFP), a molecular probe for analysis of cellular gene expression. The active chromophore of GFP from the jellyfish *Aequorea victoria* is generated via the spontaneous cyclization and oxidation of a serine–dehydrotyrosine–glycine trimer within a defined hexapeptide sequence of the protein and shows a green fluorescence with $\lambda_{\text{em}}^{\text{max}} \approx 509$ nm (153). We have utilized the advantages of GFP as a reporter gene following introduction of a deoxyribonucleotide sequence encoding the 23-mer target RNA aptamer (R23) at the 3’-end of the GFP gene prior to the stop codon (Fig. 6.1). Recognition of this R23 sequence in the transcribed mRNA results in cleavage in vivo, and the fluorescence intensity of GFP (Figs. 6.1, 6.2 and 6.3) decreases as a consequence of the destruction of translatable mRNA for GFP (156,157). Such a strategy provides a framework for the screening of novel drugs in the cleavage of pathogenic mRNA sequences.

To test whether the R23 sequence had been cleaved by copper kanamycin A in vivo, we utilized a 96-well reader format that would provide high throughout screening. The cells were grown to log phase and induced with IPTG to provide mRNA transcripts.
In contrast to eukaryotic organisms, bacterial cells are permeable to low molecular weight reagents carrying high charge densities, and so addition of such to a growth medium is followed by uptake into the cell. In our case the growth medium was supplemented with copper kanamycin A, varying from 1 to 80 mM, and following incubation at 37 °C for 4 h. The results show that translation of GFP mRNA carrying the R23 insert is significantly reduced with increasing concentration of copper kanamycin A, reflected by the decrease in fluorescence intensity. These results are consistent with \textit{in vivo} cleavage of the R23 RNA target sequence by copper kanamycin A resulting in a decrease in translatable mRNA transcript. Moreover, the significant reduction in fluorescence indicates that almost all of the mRNA transcript is inactivated. Control experiments following addition of either the metal-free ligand kanamycin A (Fig. 6.4), or with either the ligand or copper derivative against GFP lacking the R23 insert, show no change in emission intensity with increasing concentration of ligand or complex. These data eliminate the possible inhibition of translation by kanamycin A itself, or of any other influence on cellular chemistry by the ligand or complex that might prevent formation of GFP. The absence of significant inhibition by the control kanamycin A ligand over the concentration range employed is significant insofar as it demonstrates that mere inhibition of translation through binding of small molecule inhibitors to mRNA target sequences is relatively ineffective at the low concentration regimes required of a drug molecule. A strategy combining recognition, binding, and degradation of a target sequence would appear more effective. The success in this novel fluorescent assay provides an initial step for the high throughout screening pathogenic mRNA targets for drug library.
LIST OF REFERENCES


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