Title
Oligomerization of the E. coli mercury transporter MerT in non-ionic detergents

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Oligomerization of the *E. coli* Mercury Transporter MerT in Non-Ionic Detergents

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Chemistry

by

Frank Herkules

Committee in charge:

Professor Hector Viadiu, Chair
Professor Antonio De Maio
Professor Daniel Donoghue

2012
The Thesis of Frank Herkules is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2012
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LIST OF ABBREVIATIONS

Å Angstrom
AA Amino Acid
AmpR Ampicillin resistance gene
Asp Aspartic Acid
AUC Analytical ultracentrifugation
BCA Bicinchoninic acid
BM(PEG)2 1,8-Bismaleimidodiethyleneglycol
BS3 Bis(sulfosuccinimidyl) suberate
C43 Walker Strain, C43(DE3) chemically competent cells
C8E4 n-Octyl-tetraoxyethylene
CMC Critical micelle concentration
Cys Cysteine
D2O Deuterium oxide
DDM n-Dodecyl-β-D-maltopyranoside
σ Density
DMSO Dimethyl sulfoxide
DTT Dithiothreitol
E. Coli Escherichia coli
EcoRI Restriction endonuclease cleavage site
EDTA Ethylenediaminetetraacetic acid
ffR Frictional ratio
Gln Glutamine
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<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>Hg&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Reduced mercury</td>
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<tr>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Inorganic mercury in its +2 oxidation state, the most common state</td>
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<tr>
<td>Hg-C</td>
<td>Mercury-carbon covalent bond</td>
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<td><em>HindIII</em></td>
<td>Restriction endonuclease cleavage site</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IEC</td>
<td>Ion exchange chromatography</td>
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<tr>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Kanamycin resistance gene</td>
</tr>
<tr>
<td>LacI</td>
<td>Lactose inducible gene</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td><em>mer</em></td>
<td>Mercury resistance gene</td>
</tr>
<tr>
<td>MP</td>
<td>Membrane protein</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>M&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Molecular weight in Daltons</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
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<tr>
<td>OG</td>
<td>n-Octyl-β-D-glucoside</td>
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<td>pET28</td>
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<td>Phe</td>
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<td>pMAL-p2G</td>
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<tr>
<td>ppb</td>
<td>Parts per billion</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>PPS</td>
<td>PreScission protease cleavage site: Leu-Glu-Val-Leu-Phe-Gln-/Gly-Pro</td>
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<tr>
<td>Plac</td>
<td>Promoter downstream of the LacI gene</td>
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<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
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<tr>
<td>Rs</td>
<td>Stokes Radii</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>Sw</td>
<td>Sedimentation coefficient</td>
</tr>
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<td>S20w</td>
<td>Sedimentation coefficient relative to that of water</td>
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<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
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<tr>
<td>TMH</td>
<td>Transmembrane helix</td>
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<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>η</td>
<td>Viscosity</td>
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ACKNOWLEDGEMENTS

To begin with, I would like to thank Professor Hector Viadiu for his support as the chair of my committee and being an amazing mentor and P.I. I started working in Dr. Viadiu's lab as an undergraduate and it is there where I found a desire to do further research. Through his enthusiasm and passion, I have expanded my background in biochemistry and I was exposed to other individuals with similar interests within his lab.

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Lastly, I would like to thank my mother, family and friends for their love and support during my time here.
ABSTRACT OF THE THESIS

Oligomerization of the *E. coli* Mercury Transporter MerT in Non-Ionic Detergents

by

Frank Herkules

Master of Science in Chemistry

University of California, San Diego, 2012

Professor Hector Viadiu, Chair

Bacteria are able to survive environments with high concentrations of toxic ionic mercury due to their ability to reduce it to the less toxic neutral species. In order to reduce soluble mercury, bacteria use a subtle mechanism that involves, at least, three proteins. In the periplasm, MerP traps soluble mercury and passes it to a membrane transporter, MerT that introduces mercury to the cytoplasm, where a reductase uses NADPH to convert ionic mercury to the more volatile neutral mercury. The initial trapping of the mercury by MerP and its final reduction by MerA are well understood at the molecular level, however its transport to the cytoplasm by MerT is not understood at the molecular level.
We have over expressed and purified the *E. coli* mercury transporter, MerT, in milligram quantities and identified its dimeric oligomer state by three biochemical techniques: chemical cross-linking, size exclusion chromatography, and analytical ultracentrifugation.
CHAPTER ONE

INTRODUCTION
1.A. Mercury in the Environment

Mercury is a rare element with an abundance of 85 ppb by weight or 9 ppb by moles on the Earth’s crust (Lide, 2002; Morgan and Anders, 1980; Ehrlich and Newman, 2008). During the pre-industrial age, high mercury levels were limited to a few regions in the world in the form of cinnabar ores and amalgam metalloids. However, since the beginning of the industrial age, anthropogenic activities have spread higher mercury levels to pollute the air, soil and water worldwide (Figure 1.1). Currently, half of the mercury released into the environment is from manufacturing and combustion sources comprising, but not limited to, power plants, coal-fired utility boilers, municipal waste combustion, commercial/industrial boilers, and medical waste incinerators (EPA, 1997). Furthermore, a 1997 EPA report to Congress demonstrated that, from 1994 to 1995, the largest mercury emissions sources were from fossil fuel combustion utility boilers (EPA, 1997). After publication of the EPA report, emission standards have been tightened to lower the amount of mercury released into the biosphere. Specifically, medical waste incinerators and municipal waste combustors have had > 96% reduction in mercury released from 1990 to 2005. Yet power plants have only reduced their mercury emissions by 10%, within this same time frame, from 59 to 53 tons per year (EPA, 2011). Nonetheless, environmental mercury pollution continues and it is a threat to human and wildlife populations.

Mercury has many useful applications in today’s world, yet the threat to all forms of life generated by its inappropriate use or careless release into the environment cannot be ignored. Mercury can have many beneficial uses that include medicines, fluorescent lamps, cosmetics, fungicides, thermometers and disinfectants (Ball et al., 2001;
Masamitsu, 2007; MDH 2011; Englender et al., 1980; Smith, 2009; Weed and Ecker, 1931). However, many of these uses involving mercury have been or are being phased out, particularly those involving medicinal or agricultural products.

Mercury exists in three molecular states as elemental, ionic or organometallic forms that can be released into the environment. The most toxic forms are the organomercurial species that accumulate in higher predatory organisms. For example, methyl-mercury is the most common, and it has been found at toxic levels in aquatic birds, such as mallards and egrets, predatory fish, like sharks and swordfish, as well as in humans who have consumed contaminated wildlife (FDA, 2006). The two most dramatic cases of mercury poisoning in humans are the one suffered by eating shellfish and fish from Minamata Bay that had been polluted by the Chisso chemical company, from 1932 to 1968, and the one suffered by Iraqi farmers in the 1970’s when they consumed imported wheat that had been treated with methyl-mercuric fungicides by the Cargill Corporation (Harada, 1995; Bakir et al., 1973). In both communities, after consumption of contaminated foods, systemic toxic effects were observed. More specifically, the motor and sensory organs were affected such that patients presented symptoms like ataxia, insanity, birth defects and death.

As of today, it is undeniable that environmental mercury contamination continues due to the increasing combustion of fossil fuels that produce mercuric sub-products and their spread by natural geochemical cycles to areas beyond the initial contaminated sites. In spite of mercury toxicity and its widespread presence, promising implementation of bioremediation schemes have been tested using microorganisms that are able to evade
such toxic effects (Nascimento and Charton-Sauza, 2003; Nagata et al., 2006; Nagata et al., 2009; Yamaguchi et al., 2007).

1.B. Bacterial Mercury Resistance Conferred by the mer Operon

Heavy-metal toxicity has been present since life's the early evolution and, accordingly, early microorganisms developed the first heavy-metal detoxification mechanisms. These mechanisms are diverse: such as the blockage of ions from entering the cell, the presence of active ion efflux pumps codified by resistance genes, the intracellular ion sequestration by proteins, the extracellular sequestration via polysaccharides, and, lastly, the enzymatic conversion of the toxic metal to a less toxic or volatile form (Mathema et al., 2011). In the case of mercury detoxification in microorganisms, the mer operon evolved to use one or more of these methods. Moreover, because the mer operon’s ubiquitous presence in microbial organisms it is believed that the mer operon spread via horizontal gene transfer events (Lal and Lal, 2010).

The mer operon can confer a narrow or a broad spectrum mercury resistance and detoxification (Figure 1.2). For instance, a narrow spectrum is characterized by resistance to only inorganic mercury species, Hg$^{2+}$, while a broad spectrum provides resistance to both inorganic and organomercurial species, i.e. CH$_3$Hg$^+$. The mer operon involves a set of genes comprising of detection proteins, like MerR/D, transport proteins, like MerP/T/C/E/F, and enzymatic detoxification proteins, like MerA/B. Briefly, in gram-negative bacteria the pathway of detoxification begins with mercurial species diffusing past the outer membrane into the periplasm where the MerP with two exposed cysteine residues traps the mercury. The MerP is the first line of defense against Hg$^{2+}$ by shuttling
the mercuric species to the inner membrane transport proteins, and, in this manner, blocking mercury from reacting with other periplasmic proteins. MerT is the most conserved transporter and its role is to transfer mercuric species from the periplasmic MerP to the cytoplasm for enzymatic reduction. After mercury enters the cytoplasm, the mercury reductase enzyme, MerA, reduces it from Hg$^{2+}$ to Hg$^0$. MerA is considered to be the essential protein required for a bacteria to survive the presence of mercury, yet the level of resistance to mercury is increased by the complete mer operon. In organisms that have a broad spectrum resistance, organomercurials follow a similar pathway except that after the mercury species enters the cytoplasm, it is transferred to MerB that acts as a mercury lyase of the mercury-carbon bond and again the mercury is finally reduced by MerA (Mathema et al., 2011). At last, once the ionic mercury species has been reduced to its elemental form, it volatilizes out of the cell due to its high vapor pressure (Figure 1.3). Ergo, the microorganism has escaped the toxic effects of mercury.

1.C. Bacterial Mercury Transport

1.C.1. Molecular mechanism of mercury resistance

What are the molecular mechanisms that operate within the cell to resist mercury toxicity? It is known that cysteines play a critical role throughout the mercury detoxification pathway. As a consequence, mutations that replace any of the cysteines of the proteins involved in the pathway lower mercury transport efficiency and the ability of the organism to survive. Specifically, cysteines mediate the shuttling, transport, lysis, and reduction of mercuric species by using mercury's affinity towards sulfur (Schiering et al., 1991; Morby et al., 1995; Lafrance-Vannasse et al., 2009). The same affinity of
mercury for sulfur explains the toxic effect of mercury when it binds the sulfur atoms present in most proteins.

Every protein encoded by the mer operon has cysteines that are able to specifically bind to mercury. For instance, the regulatory MerR protein binds to the promoter-operator region of the mer operon (Ansari et al., 1995) and up- or down-regulation is dictated by the presence or absence of mercury binding (Yu et al., 1996). In addition, the bound and unbound structures of the periplasmic protein, MerP, have also been determined (Steele and Opella, 1997), where a mutation in Cys36 reduces the ability of MerP to pass the mercury ion to MerT (Morby et al., 1995). Furthermore, the NADPH-dependent enzymatic mechanism of mercury reduction by MerA has also been elucidated (Schiering et al., 1991), and mutations in Cys135 and/or Cys-140 abolish MerA mercury reductase activity (Moore and Walsh, 1989). For the mercury lyase, MerB, crystallographic analysis has shown that Cys96 and Cys159 involved in binding and C-Hg bond cleavage, while Asp-99 is responsible for proton transfer to the carbon substituent (Lafrance-Vannasse et al., 2009). Lastly, mutations in Cys24 and Cys25 of MerT extinguish mercury transport across the cell membrane (Morby et al., 1995). Considering all the evidence, it is undeniable that mercury transport and detoxification requires the presence of cysteine residues.

1.C.2. Remaining questions to understand the molecular mechanism of mercury resistance

Although the structures of core resistance proteins, MerA and MerB, has been determined, together with the regulatory, MerR, and the periplasmic MerP shuttling protein, the transfer of mercury from the perioplasm to the cytoplasm remains a mystery.
In gram-negative bacteria, the canonical detoxification proteins are MerP, MerT and MerA. First, MerP, a small 72 amino acid protein, is first line of defense against mercury toxicity because two cysteines in the GMTCXXC metal-binding loop bind mercury. NMR studies suggest that upon mercury binding, Phe38 in MerP forms a hydrophobic patch that might mediate protein-protein interaction with the transporter MerT (Steele and Opella, 1997). MerP relays the mercury substrate to MerT, a 116 amino acid membrane protein that transports the ion from the periplasm to the cytoplasm. From the canonical triad involved in mercury detoxification, Merp-MerA-MerT, MerT is the protein pathway with less structural information available.

Previous studies have demonstrated that MerT is capable of transporting Hg2+, even without the help of MerP (Hamlett, 1992). In the periplasmic side, Cys24 and Cys25 has been suggested to be solvent exposed, but this proposal is yet awaiting structural confirmation (Howell et al., 2005) (Figure 1.4). In addition, the kinetics and energetics of the MerT transport mechanism is not understood. In the cytoplasmic side, a loop of MerT has been implicated to interact with the N-terminal region of MerA for optimal mercury transport; this interaction does not require the cysteines present in the cytoplasmic loop of MerT (Schue et al., 2008). Interestingly, a synthetic 23 amino acid of the MerT cytoplasmic loop is capable of specifically transferring mercury to MerA (Rossy et al., 2004). Albeit slowly, the putative cytoplasmic interactions are being elucidated, yet how MerT mediates transport across the membrane remains to be determined. During the last step in the mechanism, MerT transfers the mercury ion to the N-terminal domain of MerA. Where the mercury reductase, MerA, functions as a dimer with two domains per monomer: the N-terminal, MerP like metallochaperone domain that
interacts with MerT, and 565 amino acid catalytic core. Within the catalytic core, Cys135 and Cys140 bind the toxic Hg\(^{2+}\) ion to convert it to the less toxic and volatile Hg\(^0\) form by the following enzymatic reaction: \(\text{NADPH} + \text{Hg}^{2+} \rightarrow \text{Hg}^0 + \text{NADP}^+ + \text{H}^+\) (Moore and Walsh, 1989). Once reduced, the elemental mercury species volatilizes out of the cell due to its high vapor pressure and neutral form that makes it more permeable to the hydrophobic membrane. As mentioned before, the mercury-protein interactions of MerP and MerA have been elucidated, but not for the transmembrane transporter MerT.

1.C.3. Membrane mercury transporters

How are the membrane mercury transport proteins MerT/F/E/C organized? These transporters are the most elusive component of the mercury detoxification pathway simply because they are intrinsic membrane proteins. To begin with, the oligomeric structure and kinetics of transport for these transporters still have not been determined. It is thought that because of MerT's small size, 116 amino acids, that it functions as a multimer, like many other bacterial transporters (Morby et al., 1995). It is thought that MerT, and its homolog proteins, function as a channel transferring the mercuric species from the Cys24 and Cys25, adjacent cysteines in the first transmembrane helix, to the two cysteines in the cytoplasmic region, Cys76 and Cys-82 (Yamaguchi et al., 2007). If a channel is formed, how many monomers are needed to form it and would all the transporters have all the same multimeric state? On the other hand, multimerization might differ from one homolog to the other because each transporter has its own specificity, as MerE favorably transports methyl/ionic mercury and MerT phényl/ionic mercury (Sone et al., 2010). Incidentally, cysteines within MerT seem not to be necessarily required for phenylmercury transport by MerT (Kiyono et al., 2000). Could transporters have more
than one channel? And, if so, what is their oligomer organization? There is little information on the organization of this membrane protein transporter other than putative helices derived from Kyte-Doolittle hydropathicity plots ranging from 2-4 transmembrane helices. MerE/F has been reported to contain two domains spanning the cell membrane, while MerT contains three. MerC is believed to be the latest evolution of the membrane transporters with four membrane spanning domains.

1.B. Objective of Thesis

The objective of this thesis is to have a better understanding of the organization present in the E. coli mercury transport protein MerT in non-ionic detergents and include these findings for the crystallization of MerT:

1) Oligomerization of MerT in detergents will be probed by chemical cross-linking of putative solvent exposed primary amines and thiol groups present in MerT

2) Oligomerization will be investigated for MerT/detergent complex formation by Size Exclusion Chromatography (SEC) and those complexes indicate a local range for MerT oligomers

3) Lastly, MerT multimers will be investigated for their complex size and elucidate the MerT multimer in detergents by Analytical Ultracentrifugation (AUC) Sedimentation Velocity (SV) experiments with the gravitationally transparent C₈E₄.
Figure 1.1. Mercury fate, transport and exposure. The largest releases of mercury into the biosphere from anthropogenic activities are highlighted by fossil fuel combustion and power plants. Moreover, the geochemical cycling of mercury beyond its site of origin contributes to the global mercury pollution where wildlife and people are at risk. The largest threat to humans and wildlife originates from the formation of organomercurials created by microorganisms, namely the production of methyl mercury as another form for mercury detoxification, not discussed here. Schematic is an adaptation from http://www.mercury.utah.gov/.
Figure 1.2. Schematic representation of the *mer* operon present in gram-negative mercury resistant bacteria. Mercury detection/regulatory proteins, MerR/D, are labeled in white; periplasmic and membranous transport proteins, MerP/T/C/E/F, are seen in black; and, lastly, enzymatic detoxification proteins are seen in blue.
Figure 1. Broad spectrum resistance and detoxification of gram-negative bacteria containing the mer operon. 1) MerP protects the periplasmic proteins by binding to mercury and shuttling it to the membrane transporters. 2) Membrane mercury transporters, MerT/E/F/C, mediate mercury transport across the cell membrane. 3) After the mercury species enters the cytoplasm, one of two enzymes uptake the substrate. 4) Organomercurials are relayed to MerB for lyase of the C-Hg bond producing inorganic mercury. Inorganic mercury is transferred to MerA for enzymatic conversion of Hg^{2+} to Hg^{0}. 5) Elemental mercury, because of its high pressure and neutrality, volatilizes out of the cell. The crystal structure for the periplasmic and cytosolic proteins has been included.
Figure 1.4. Expected transmembrane topology for MerT. Expected transmembrane domains from previous studies involving hydropathicity plots. Cysteine residues required for the efficient transport of mercury are labeled in red while transmembrane domains are labeled in blue boxes.
References


CHAPTER TWO

EXPRESSION AND PURIFICATION OF MerT
2.A. Introduction

Membrane proteins (MP) comprise only 1% of the total number of the solved structures deposited in the Protein Data Bank. In contrast, MP's constitute 20-30% of the proteins in most organisms, and 60-70% of drug targets (Lundstrom 2004). The elucidation of MP structures is key to the development of new drugs and to the understanding of signaling mechanisms occurring in the cell membrane. However, major difficulties must be overcome to determine its structure because they are mostly hydrophobic and reside within the cell membrane. Besides, they must be isolated from other MP in high enough amount and purity.

To structurally investigate MPs, one needs to focus on those that naturally occurring large quantities, like rhodopsin, or to over express the target protein in a heterologous organism. Since few MP's normally occur in enough high concentration to be isolated from the natural source, recombinant over expression of the target proteins with an affinity tag is typical method to obtain the large concentration of MP required to carry structural work. (Niegoski et al. 2006) However, there is still another obstacle that must be overcome, the isolation of pure, functional proteins from the host's MPs. To preserve the structure and functionality of MP’s with exposed hydrophobic side chains, it is required to use detergents for the efficient disruption of the cell’s membranous fraction. For this reason, the choice of detergents is essential such that it will determine whether the protein will retain its functionality. Detergents facilitate the solubilization of MPs to ease purification, particularly when the protein has an affinity tag (Figure 2.2). There are three main classes of detergents that are typically used for MP isolation: ionic, non-ionic and zwitterionic. The class of detergent that has been the most successfully used for
structural elucidation is the non-ionic group (Figure 2.1) (Newstead et al., 2008). Furthermore, the last impediment to structure determination is the organization of detergents around the membrane protein.

A further complication to study MPs is the nature of the interaction of the detergent with the protein. Detergents are critical in the screening of conditions for the crystallization of MPs, a process where a balance between the protein and the detergent must be considered in combination with the addition of solutions that promote protein precipitation (Newstead et al., 2008). An alternative to growing 3D crystals is 2D crystallization where the MP is reconstituted into lipids and its crystallization is analyzed with the electron microscope; yet this method is hindered by the difficulty to remove the detergent bound (Hasler et al., 1998). Another alternative method to determine MP structure is NMR, but it is most effective with small membrane peptides (Montaville and Jasmin, 2010).

2.B. Detergents

As mentioned earlier, detergents play a critical role in the isolation of functional MPs. An important phenomenon is that at concentrations above the critical micelle concentration (CMC) detergents disrupt through the intercalation of detergent molecules into the lipidic membrane. The lipidic membrane dissolves forming micelles of detergents, lipids, and proteins. Thus, the formation of micelles more readily solubilizes proteins and eases its purification. Hence, the choice of detergents is essential; particularly the use of non-ionic, instead of ionic, detergents, because proteins retain their
function and their structure as crystal structures have shown (Figure 2.1) (Newstead et al., 2008; Jasti et al., 2007; Yamashita et al., 2005).

In this thesis, I have used the following detergents: n-octyl-tetraoxyethylene (C8E4), n-dodecyl-β-D-maltopyranoside (DDM) and n-octyl-β-D-glucopyranoside (OG) (Figure 2.3), all of which have been useful to determine crystal structures of MPs.

Detergents were purchased from Bachem for C8E4 and from BioWorld for DDM and OG. DDM and OG detergents were maintained as 25% (w/v) stocks in water while C8E4 detergent was maintained in 100% stock until use.

2.C. Cloning of MerT

The mercury transporter MerT gene from E. coli coding for 116 amino acid polypeptide was cloned into pMAL-2pG vector using EcoRI and HindIII cloning sites. The ligated plasmid was then sequenced and transformed into E. coli C43 cells. A Prescission protease site was inserted between the N-terminal MBP (Maltose Binding Protein) tag, such that MerT could be isolated by amylose affinity purification and then cleaved from the tag. Cleavage of the MBP tag by Prescission protease is between the Gln and Gly residues in the recognition sequence: LEVLFQ/GP. In a different construct, the MerT gene was also cloned into a pET28 expression vector with an 8-His tag at the N-terminus using the same cloning sites as in pMAL vector, but without the protease site (Figure 2.4 and 2.5).
2.E. MerT Expression

An overnight 5 ml culture of *E. coli* C43 cells containing the over-expression vector carrying the *merT* gene were added to a 1 liter of Luria-Bertani (LB) medium at 37°C. At an OD$_{600}$ ~ 0.6-0.9, 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. Protein expression was conducted for ~ 6-12 hours at 25°C. Then, cells were pelleted by centrifugation at 3000 x g and the cell pellet was lysed for protein purification or stored at -20°C until it was lysed.

2.F. Cell Lysis

The cell pellet was re-suspended in 30 ml of Buffer A (200 mM NaCl and 25 mM Tris pH 7.5). In addition, lysozyme (5 mg/g cell pellet) and phenylmethylsulfonyl fluoride (2 mM PMSF) were included in the re-suspension and it was incubated on ice for 1 hour prior to lysis. Cells were lysed via French Press (1000 PSI) maintaining the lysate on ice. The lysate was centrifuged at 30,000 x g to pellet membranous fraction and unbroken cells. The pellet with the membranous fraction was re-suspended with Buffer B (25 mM Tris pH 7.5, 800 mM NaCl, and 2 mM EDTA) for another round of homogenization followed by centrifugation at 30,000 x g (Figure 2.6, Lanes 2-4).

2.G. Solubilization of the Membranous Fraction

The pellet with the membranous fraction was solubilized with Buffer C (40 mM DDM or 40 mM OG, 25 mM Tris pH 7.5, and 200 mM NaCl) for 2 hours at room temperature or 12-16 hours at 4°C. The solubilized membranous fraction was centrifuged once more at 25,000 x g to remove cell debris and membranes that were not solubilized.
The soluble fraction was then subjected to affinity purification (Figure 2.6, Lanes 5 and 6).

2.G.1. Isolation of MBP-PPS-MerT

The solubilized lysate fraction was incubated with amylose resin for batch binding for 1 hour at 4°C. The resin with the bound protein was then passed through a gravity column, and resin was washed with Buffer A (100 times the resin volume (column volume - cv)). The fusion protein was eluted with 5 x cv of Buffer D (50 mM Maltose, 20 mM Tris pH 7.0, 20 mM NaCl, 0.25 mM TCEP, 0.25 mM EDTA, 0.5 mM DTT, 0.98 mM DDM, and 2% (v/v) glycerol). The MBP tag from fusion protein was cleaved using Prescission protease (1 U/100μg of fusion protein) for 12-16 hours at 4°C. The digested sample was then subjected to Sepharose-Q ion exchange chromatography (IEC) for MBP tag removal (IEC-Buffer A: 20 mM Tris pH 7.5, 10 mM NaCl, 0.98 mM DDM, 0.25 mM TCEP, 2% (v/v) glycerol; IEC-Buffer B: 20 mM Tris pH 7.5, 1 M NaCl, 0.98 mM DDM, 0.25 mM TCEP, and 2% (v/v) glycerol). Isolated MerT was concentrated to ~1 ml and stored at 4°C until use for size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC). Purification was analyzed by SDS-PAGE (Figures 2.6-2.8) and quantified using the BCA Protein Assay Reagent (Thermo).

2.G.2 Isolation of 8His-MerT

The solubilized lysate fraction was incubated with Ni-NTA resin for batch binding for 1 hour at 4°C. The resin with the bound protein was then passed through a gravity column and the resin was washed with Buffer A (50 x cv). Moreover, resin was washed with 20 x cv Buffer E (10-80 mM imidazole, 20 mM Na₂HPO₄ pH 7.0, 100 mM NaCl, 0.25 mM TCEP, and 0.98 mM DDM). The target 8His-MerT was
eluted with 200 mM imidazole added to Buffer E. Furthermore, the pure MerT was concentrated to ~1 ml and stored at 4°C until it was used for cross-linking. Purification was analyzed by SDS-PAGE (Figure 2.10) and quantified using the BCA Protein Assay Reagent.

2.I. Validation of protein identity by SDS-PAGE, western blotting and mass spectrometry

Samples at each purification step were collected and analyzed by SDS-PAGE. Furthermore, Sepharose-Q pure MerT was concentrated to concentrations higher than 10 mg/mL. The buffer was exchanged to Mass Spec sample buffer (20 mM Tris pH 7.5, and 100 mM NaCl) and diluted to 1 mg/ml. A 10 μl aliquot was then sent to Tufts Mass Spec facility for analysis (Figures 2.6-2.9).
Figure 2.1. Detergents used in the crystallization of membrane proteins and the families those membrane proteins belong to. Data and diagrams are from Newstead et al. 2008.
Figure 2.2. Schematic representation of the solubilization process for membrane protein purification. The filled in circles with tails represent detergents while the empty circles with tails represent the lipids in the membranous fraction. Diagram obtained from Rigaud and Levy 2003.
Figure 2.3. The three non-ionic detergents used for the purification and characterization of MerT. A. The polyoxyethylene used detergent, n-octyl-tetraoxyethylene (C₈E₄), used in SEC and AUC experiments. B. The alkyl glucopyranoside, n-octyl-β-D-glucoside (OG), used in SEC and screening trials. C. the alkyl maltopyranoside, n-dodecyl-β-D-maltopyranoside (DDM), used in SEC and screening trials.
Figure 2.4. Experimental Outline. Each step for the expression, isolation and analysis of MerT is given above.
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**Figure 2.5. Linear representation of vector sequences generated.** Each plasmid was transformed into *E. coli* C43 cells for expression of target proteins.
Figure 2.6. Sequence alignments and expected locations of cysteine pairs present in \textit{MerT}. Labeled red dots represent cysteines. Predicted transmembrane helices are designated with a black overlines in the sequence and blue rectangles in the diagram. Mutants included were used for EXAFS experiments that are awaiting access to the synchrotron time to be carried out.
Figure 2.7. SDS-PAGE analysis on the purification of MBP-PPS-MerT. Isolated fusion MBP-PPS-MerT protein can be observed in the last lane on the right hand side. Isolated fusion protein was obtained via 50mM maltose elution.
Figure 2.8. Sepharose-Q IEC of cleaved MBP-PP-MerT. MBP is the peptide that is bound to column while the cleaved merT passes through the column, P1. P2 corresponds to cleaved MBP and P3 to any other MBP/fusion tightly bound to the column.
Figure 2.9. SDS-PAGE and Western blot of Sepharose-Q IEC purified MerT. A. SDS-PAGE shows P1 contains the cleaved MerT product. There are two additional bands above the expected M\textsubscript{W} which might represent a dimer or trimer. P2 demonstrates a protein for the expected cleaved MBP tag and P3 corresponds to tightly bound MBP and undigested fusion protein. B. Western blotting, using a α-MBP anti-body conjugated to HRP, confirms the removal of the MBP tag in P1. Cleaved MBP and undigested fusion protein can be seen in P2 and P3.
Figure 2.10. MS of IEC purified MerT. Sample was sent to Tufts Mass Spec Facility for analysis. The highest peak at 13009 kDa corresponds to that of MerT, expected was 13008 kDa.
Figure 2.11. SDS-PAGE and Western blot on the purification of 8His-MerT.
A. SDS-PAGE purification of 8His-MerT; 8His-MerT is present in the far right two imidazole elutions with an apparent M₆ of ~15kDa. B. In the Western blot, 8His-MerT is also present in the soluble fraction and the pellet that corresponds to insoluble aggregates. The Western blot was developed using α-His anti-body conjugated to HRP.
References:


CHAPTER THREE

CHARACTERIZATION OF MerT OLIGOMERS VIA CROSS-LINKING
5.A. Introduction

Cross-linking is a technique that aims to chemically join two closely interacting molecules. Cross-linkers have chemically reactive ends to specific functional groups that are present in proteins, such as amines, sulfhydryl, carboxyl, etc. To probe for possible multimers of MerT, homobifunctional cross-linking reagents, like BM(PEG)$_2$ and BS$_3$, were used to exploit exposed thiol and primary amines in the N-terminus and the loop of MerT.

Since the MerT cytoplasmic loop contains many functional groups (-OH, -SH, -COOH, and -NH), the use of homobifunctional cross-linking reagents was implemented to reduce the possible formation of non-specific self cross-linking so that only desired residues may be reacted. More specifically, MerT hydrophobicity plots show that there are exposed cysteines, Cys76 and Cys82, on the cytoplasmic loop of MerT (Yamaguchi, 2007; Morby, 1995). Using BM(PEG)$_2$ that has a 14.7 Å spacer arm length, might avoid that residues in the same monomer from reacting and conjugation across monomers could be observed upon cross-linking (Figure 5.1 and 5.3). Along the same lines, the cross-linking of Lys67, Lys77 and Lys88 in the same cytoplasmic loop of the same monomer could be avoided by using BS$_3$, a primary amine reactive with another long 11.4 Å spacer between reactive groups (Figure 5.2 and 5.3). Quick analysis for possible covalent bond formation was performed by SDS-PAGE.
5.B. Methods

5.B.1. BM(PEG)_2 Cross-linking

Stock 5 mM BM(PEG)_2 in 5% (v/v) DMSO was then diluted to final working conditions of 0.25% (v/v) DMSO. Isolated 8His-MerT was brought to concentrations higher than 10 mg/ml and incubated with 5 mM TCEP prior to cross-linking. The MerT protein was diluted to 0.038 mM (0.5 mg/ml) in Buffer G (20 mM NaH_2PO_4 pH 7.0, 100 mM NaCl, 0.98 mM DDM, and 0.25 mM TCEP) and BM(PEG)_2 was added in incremental stoichiometric amounts. Reactions were incubated for 2 hours at room temperature and quenched with a final concentration of 5mM DTT. Samples were then analyzed via SDS-PAGE gels (Figure 5.4).

5.C.2. BS^3 Cross-linking

A working stock solution of 5 mM BS^3 in water was prepared. Isolated 8His-MerT was brought to concentrations higher than 10 mg/ml and incubated with 5 mM TCEP prior to cross-linking. The MerT protein was diluted to 0.038 mM (0.5 mg/ml) in Buffer G and the BS^3 stock solution was added in incremental stoichiometric amounts. Reactions were incubated for 2 hours at room temperature and quenched with a final 5 mM Tris concentration. Samples were then analyzed via SDS-PAGE (Figure 5.5).

5.E. Results of X-linking

Oligomerization of MerT was observed for the two cross-linkers used. SDS-PAGE demonstrated that cross-linking with BM(PEG)_2 produced M_w shifts from a monomer to higher order oligomers; the most obvious shift was to dimeric species with minute amounts corresponding to a trimer and, possibly, a tetramer (Figure 5.4). While
the theoretical M\textsubscript{W} of the 8His-MerT is \(~14\text{kDa}, the apparent M\textsubscript{W} of MerT prior to any cross-linking, in SDS-PAGE, is slightly higher than the \(~15\text{kDa marker}. The observed M\textsubscript{W} shifts corresponded according to the protein markers to dimeric, trimeric and tetrameric species. In addition, similar results were observed for cross-linking reactions with BS\textsuperscript{3}, although only a single higher order oligomer, corresponding to a dimer, was observed (Figure 5.5).

5.E. Discussion

All the experimental conditions that we tested in the cross-linking experiments conclude that MerT in detergents is a multimer. The addition of increasing stochiometrical amounts of BM(PEG)\textsubscript{2}, definitely demonstrated that a second dimeric species is embedded in the DDM micelles. Prior to any cross-linking, the gel show a very faint and miniscule signal from a M\textsubscript{W} species other than the monomer, a possible dimer. Then with the addition of the cross-linker, at one fifth the concentration of the protein present, this second species becomes more prominent and indication that a dimer is present (Figure 5.4 Lane 3). However, there is the possibility of the existence of other multimers besides the observed dimer, namely trimers and tetramers. The formation of a trimer is most noticeable when BM(PEG)\textsubscript{2} and MerT are in equal amounts (Figure 5.4 Lane 5), and, the formation of a tetramer only discernable once BM(PEG)\textsubscript{2} is about five-fold more plentiful than MerT (Figure 5.4 Lane 7). Yet, both trimer and tetrameric multimers are only a fraction of the observed monomer and dimer species. Increasing the amount of BM(PEG)\textsubscript{2} did not increase the proportion of trimer or tetramer oligomers observed, but it did increase the likelihood of aggregation as observed in the top of the
gels or precipitation during the reaction. It is very likely that the formation of trimers and tetramers to be the consequence of non-specific cross-linking when the cross-linker might have reacted with the vicinal cysteines present in MerT. We are trying to confirm this possibility by testing cross-linking reaction in proteins carrying serine mutations instead of the cysteines.

Contrary to the multiplicity of oligomers observed with BM(PEG)$_2$ cross-linking, BS$_3$ cross-linking only yielded dimers. In an attempt to verify the oligomerization observed with thiol reactive cross-linkers, I tested the reactivity towards cross-linkers of residues having primary amines. Like BM(PEG)$_2$, BS$_3$ produced dimer oligomers at concentrations lower than MerT and, like BM(PEG)$_2$, dimer-monomer signals remained relatively the same from five-fold less to five-fold more cross-linker compare to MerT concentration (Figure 5.5 Lanes 3-7). Interestingly, there appears to be the formation of a trimer species, though very faintly (Figure 5.5 Lane 3). But this higher order oligomer is not observed at increasing amounts of BS$_3$ (Figure 5.5 Lanes 4-7), and I assume the trimer to be an artifact of the sample ran. Furthermore, unlike BM(PEG)$_2$, BS$_3$ cross-linking reactions did not show tetrameric multimer formation. Thus cross-linking with BS$_3$, supports the possibility for the natural formation of only MerT dimers in the membrane. Considering that the all the target residues for BS$_3$ lie on the same face of MerT, this could explain why only the dimer oligomer was observed. Whereas, BM(PEG)$_2$ would allow for reactions to occur in both faces of the transporter and yield more non-specific oligomerization.
Figure 3.1. BM(PEG)$_2$ reaction with sulfhydryl containing compounds. Scheme for conjugation between maleimide-activated cross-linker and sulfhydryl containing peptides for the formation of a stable, non-reducible thioether bond. Diagram adapted from Thermo #22336.
Figure 3.2 BS\textsuperscript{3} reaction with primary amine containing compounds. Scheme for conjugation between amine-reactive N-hydroxysulfosuccinimide (NHS) cross-linker and primary amine containing peptides for the formation of a stable amide bond. Diagram adapted from Thermo #25420.
Figure 3.3. Putative sites for covalent chemical crosslinking. A. Sulfhydryl groups of cysteines (Cys-24, Cys-25, Cys-76 and Cys-82) are indicated by red circles, where possible cross-linking with BM(PEG)$_2$ may occur. Vicinal cysteines, Cys-24 and Cys-25, have been indicated for possible cross-linking if they are solvent exposed. B. Primary amine residues (N-terminus, Lys-67, Lys-77 and Lys-88) are indicated by orange circles where possible cross-linking with BS$_3$ might occur.
Figure 3.4. Cross-linking of 8His-MerT with BM(PEG)$_2$. Incremental stoichiometric amounts of BM(PEG)$_2$ were added to MerT (MerT:BM(PEG)$_2$). Oligomer formation for MerT was observed ranging from the non-cross-linked monomer to a possible tetramer. The most discernible oligomer obtained was that of a dimer (Lanes 3-7) with possible higher order oligomers (Lanes 6 and 7).
Figure 3.5. Crosslinking of 8His-MerT with BS$_3$. Incremental stoichiometric amounts of BS$_3$ were added to MerT (MerT: BS$_3$). The most discernible oligomer obtained was that of a dimer (Lanes 3-7) with no higher order oligomers observed. A possible trimer (Lane 1) was observed, but not in later reactions which might be an artifact of the gel or sample ran.
References:


CHAPTER FOUR

ANALYSIS OF PURIFIED MERT VIA SIZE EXCLUSION CHROMATOGRAPHY
3.A. Introduction

Size exclusion chromatography is typically one of the first steps used in biochemistry to characterize biomolecules because it provides a good estimate of the molecular weight ($M_W$) of the molecule, while maintaining its biological activity. As the sample passes the resin, its migration is mainly dependent on the hydrodynamic or Stokes radius ($R_S$) of the molecule. Typically for MPs, there will only a single species observed in size exclusion chromatography that accounts for the detergent-protein complex (Kunji et al., 2008). The size of the complex depends on the amount of detergent bound to the MP. In addition, the determination of the $M_W$ will depend on comparing the sample's retention time with set standards with predetermined properties like $M_W$ and $R_S$.

3.B. Methods

3.B.1. Calibration of S200 Column

A 30ml Superdex 200 10/300 GL chromatographic separation column (GE Healthcare) was equilibrated with Buffer F (20 mM Tris pH7.5, 100 mM NaCl, 0.25 mM TCEP, 0.98 mM DDM, and 2% (v/v) glycerol). The following standards were injected into the column: blue dextran ($M_W$ = 2000 kDA), thyroglobulin ($M_W$ =669 kDa, $R_S$ = 8.6 nm), ferritin ($M_W$ = 449 kDa, $R_S$ = 6.3 nm), BSA ($M_W$ = 66.3 kDa, $R_S$ = 3.5 nm), chymotrypsin ($M_W$ = 25 kDa, $R_S$ = 3.0 nm) and imidazole ($M_W$ = 0.68 kDa) (Le Maire et al. 2008). Gel filtration retention times were monitored with a Water 486 Tunable Absorbance Detector and recorded with Clarity Lite detection systems (Figure 3.1).
3. B.2. SEC of Purified MerT

Column was equilibrated with 20 mM Tris pH 7.5, 100 mM NaCl, 0.25 mM TCEP, and 2% (v/v) glycerol, plus the selected detergent for the given experiment (DDM, OG, or C₈E₄). Detergents concentrations were kept at 1.5 to 6 times the detergent CMC. MerT, isolated from Sepharose-Q IEC, was injected into column (0.2-1.0 ml) and fractions of 700μl were collected.

3.C. Results

When a detergent is solubilized in water it will start forming micelles at a certain detergent concentration that it is unique for each detergent and it is referred to as its the critical micelle concentration (CMC). The detergent micelles formed will typically have a Gaussian distribution in size that fluctuates depending on hydrophobic interactions present in solution. To understand the oligomerization and size distribution of MerT/detergent complexes, I ran SEC experiments with three different detergents.

SEC of the MerT/DDM complex displayed a monodisperse species that was dependent on the amount of DDM present in solution. The size of an empty micelle at the CMC for DDM, 0.15 mM (Arnold and Linke, 2008), fluctuates in a range of M_w,DDM ~ 40-90 kDa (Kaufmann et al. 2006). In SEC with DDM at 0.98 mM (6 x CMC), a monodisperse MerT/DDM complex was observed with a R_s of 3.88 nm and M_w was 76 kDa (Figure 3.2). To investigate the MerT oligomer contribution to this complex, the concentration of DDM was halved to 0.49 mM (3 x CMC). This detergent concentration maintained a monodisperse particle distribution (Figure 3.2), yet the MerT/DDM complex size decreased to R_s of 3.60 nm and M_w was 59 kDa, a reduction of 0.20 nm or
17 kDa. The MerT/DDM complex size was dependent on the amount of detergent present in solution.

For the SEC of the MerT/OG complex, there was a range of possible multimers observed the initial DDM detergent was exchange for OG and with subsequent SEC runs a same multimer was observed. The size of an empty micelle at the CMC for OG, 20 mM, is ~ 25 kDa (Arnold and Linke, 2008). For screening crystallization conditions for MerT in 30 mM OG (1.5 x CMC), the MerT sample was subjected to SEC for the removal of initially bound DDM. The first injection to exchange detergents showed two peaks with the first peak corresponding to a \( R_s \) of 3.80 nm and \( M_W \) of 65.3 kDa and the second peak a \( R_s \) of 3.20 nm and \( M_W \) of 39 kDa. A to observe a single major species. The MerT/OG SEC profiles showed a smaller MerT/ subsequent SEC run of the previous sample showed a MerT/OG complex with a species similar to that of the second peak in the first injection with a \( R_s \) of 3.20 nm and \( M_W \) of 38.6kDa. To further investigate the oligomerization of MerT in OG, a final, third run of the MerT/OG complex was performed obtaining a \( R_s \) of 2.94 nm and \( M_W \) of 29.8 kDa (Figure 3.3). The substitution of OG for DDM in the MerT/DDM complex required multiple SEC runs detergent complex than what was observed for MerT/DDM.

When a third detergent was used to study MerT/detergent oligomerization by SEC analysis, the DDM was exchanged for \( \text{C}_8\text{E}_4 \) and it showed a single monodisperse species. At the CMC of \( \text{C}_8\text{E}_4 \), 7mM, the size of an empty \( \text{C}_8\text{E}_4 \) micelle is ~26 kDa (Arnold and Linke, 2008). Here, the MerT detergent exchange was conducted at 20mM \( \text{C}_8\text{E}_4 \), roughly three times the CMC. Unlike when OG was exchanged for DDM where a smaller particle was observed after exchange, the \( \text{C}_8\text{E}_4 \) substitution for DDM in the MerT/DDM complex
showed a particle that was roughly the same size of the MerT/DDM complex with $R_s$ of 4.03 nm and $M_W$ of 79.5 kDa. A subsequent run of this sample in SEC retained the same $R_s$ and $M_W$.

3.D. Discussion

Three different detergents were used for the characterization of the multimeric MerT in detergents. Our data shows that there is a variety of possible MerT/detergent complexes. The detergent that demonstrated the largest MerT/detergent complex was MerT/C$_8$E$_4$ with MerT/DDM having a slightly smaller complex, and, lastly, the MerT/OG complex being the smallest. In the SEC experiments of MerT/C$_8$E$_4$ complex, the size of the eluting particle was radically different from the empty micelle size, where the expected micelle size ~26 kDa and the observed size of the MerT/C$_8$E$_4$ complex is 79.5 kDa (Figure 4.4). In addition, the $R_s$ of the MerT/C$_8$E$_4$ complex was slightly larger than that of the MerT/DDM by ~0.1 nm so essentially the detergent exchange did not alter overall size of the MerT/detergent complex, where any difference can be attributed to the individual detergent interaction with MerT. However, this did not appear to be the case for the MerT/OG complex, where the complex size migrated with a $R_s$ of 3.80 nm followed by a second species with a $R_s$ of 3.21 nm (Figure 4.3). The former $R_s$ of 3.80 nm is characteristic of the MerT/DDM complex and, most likely, MerT/DDM complex that had not completely substituted for the OG. In addition, this higher molecular size complex is nearly absent in the second run through SEC column. Yet the second peak with $R_s$ of 3.21 nm is retained and shifted to a $R_s$ of 2.94 nm after the third SEC run. The size of the particle decreases from 39 kDa in the second peak observed in the first
injection that is retained in the second injection, to 30kDa in the third injection. The effect of exchanging detergents causes the MerT/OG complex to migrate closer to the empty OG micelle size of 25kDa. It is noteworthy to mention that the OG detergent concentration is ~1.5 times the CMC, whereas the C₈E₄ and DDM detergent concentrations were about three times or more the CMC. Being so close to the CMC of OG, the SEC experiments appeared to disrupt the homogeneity of the sample as was seen in the asymmetric peak of the elution profile. Lastly, the existence of a detergent contribution to the size of the MerT/detergent complexes is the SEC experiments at lower detergent concentrations that show lower molecular sizes, like the one for the MerT/DDM complex.

To test the MerT oligomerization and improve the possibility of crystallizing MerT, the DDM detergent concentration was lowered. The SEC profile for the MerT/DDM complex shifted from a larger to a smaller complex via halving the DDM concentration, from 0.98mM to 0.49mM. Yet, lowering the DDM concentration did not alter monodisperse nature of the SEC elution profile (Figure 4.2). Thus, the manner of which the individual detergents interact with MerT display varying micelle sizes. When MerT/detergents complexes are near the CMC, the homogeneity of the complex is altered, probably due to the lower detergent concentration affects the exposure of the hydrophobic proteins to the water in the solution. In addition, lowering the detergent near the CMC drives the MerT/detergent complex closer to the size of the empty micelle size as was seen for the OG and DDM detergents. This effect of was not directly observed for C₈E₄, because the detergent was not lowered near the CMC. However, as seen in the following chapter, lowering the C₈E₄ concentration for analytical ultracentrifugation
experiments caused a possible shift in multimers for the MerT/C₈E₄ complex from a monomer to a dimer. From these experiments, it is seen that MerT maintains a stable complex with DDM, even when its concentration is lowered closer to the CMC. This helps to determine the sole contribution MerT oligomer to the micelle size.

Based on the SEC results obtained, the oligomerization of MerT in detergents ranged from possible dimers to hexamers. MerT in DDM displayed a possible pentamer oligomer of 65 kDa in 0.98 mM DDM and a tetramer of 52 kDa in 0.49 mM DDM. In addition, MerT in C₈E₄ gave rise to a possible hexameric species of 78kDa in 20 mM C₈E₄. While MerT in OG suggested possible of dimers and trimers, 26 kDa and 39kDa respectively. In order to better assess the multimerization of MerT in detergents, C₈E₄ was used for AUC so as to not exclude the possibility higher ordered oligomers that may occur with MerT in OG, where the maximum would correspond to a trimer.
Figure 4.1. Calibration of S200 column. Ranges for MerT-det complexes are indicated within the dashed lines.
Figure 4.2. Membrane proteins solubilized in detergents. The apparent size of the protein-detergent complexes are dictated by the amount of the detergent monomers associated with the protein.
Figure 4.3. SEC of MerT-DDM complex with two different [DDM]. MerT demonstrates a monodisperse eluting species with $M_W = 78\text{kDa}$ and $59\text{kDa}$ for 0.98mM and 0.49mM DDM, respectively.
Figure 4.4. SEC of MerT detergent exchange to OG. 1st sample loaded was MerT-DDM from IEC. 2nd and 3rd pass was performed with 1st pass and 2nd pass eluent, respectively. Peak max that coincided for all three corresponded to $M_W \sim 30\text{kDa}$. 
Figure 4.5. SEC of merT detergent exchange to C8E4. 1st sample loaded was MerT-DDM from IEC. Peak max that coincided for both runs corresponded to M_W ~ 79kDa.

<table>
<thead>
<tr>
<th>Table 4.1 Summary of SEC results of MerT in three non-ionic detergents</th>
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<tbody>
<tr>
<td><strong>Detergent</strong></td>
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<tr>
<td>----------------</td>
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<tr>
<td>DDM</td>
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<td>OG</td>
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<tr>
<td>C8E4</td>
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References:


CHAPTER FIVE

CHARACTERIZATION OF MERT BY SEDIMENTATION VELOCITY
4.A. Introduction

Analytical ultracentrifugation (AUC) is a technique that monitors the sedimentation of particles under a centrifugal force. Particles under this centrifugal force sediment at a speed that is dependent on their size and shape. In particular, in sedimentation velocity experiments, the rate of sedimentation of a particle gives insight to the homogeneity, oligomerization, and aggregation state of a molecule, and as well as the relative distributions of each state (Lebowitz et al., 2002).

In sedimentation velocity experiments, the protein sample is spun at high speeds, and the protein under the high centrifugal force rapidly sediments towards the bottom of the rotor. As the sample depletes from the meniscus, a concentration boundary forms and moves as a function of time, until ultimately the sample reaches the bottom of the cell. The sedimentation process for a given molecule, or complex, can be described as a sedimentation coefficient (s) that is dependent on a set of given parameters. These molecular parameters relate to s in the Svedberg equation:

\[ s = \frac{u}{\omega^2 r} \]

The s value of a particle depends on the observed radial velocity (u) divided by the centrifugal force experienced by the particle (\( \omega^2 r \)). Where \( \omega \) is the angular velocity of the rotor and \( r \) is its position from the center of the rotor at the time of the scan. In addition, the sedimentation of the particle can, also, be described as a property dependent on the molar mass of peptide (M), its partial specific volume (\( \psi \)), the density of the solvent (\( \rho \)), Avogadro's number (\( N_A \)) and the frictional ratio (\( f/f_0 \)) which the molecule encounters while it sediments. Similarly, another manner of determining the s value is by
multiplying the diffusion coefficient of the particle (D) by its buoyant mass M(1- ρ) and dividing it by the temperature (T) and the gas constant (R) (Lebowitz et al., 2002).

However, there is a caveat when determining the s value of membrane proteins because the detergent present in the membrane protein preparation can contribute to the sedimentation of the particle. In some cases, the detergent used has a higher density than that of the solvent and the s-value increases or the density might be lower where floatation might occur and the s value decreases (Fleming, 2005). Yet detergent contribution can be circumvented with the use of neutrally buoyant detergents that are gravitationally transparent or with the tandem analysis by interference optics of dense detergents with a known refractive index contribution. In using neutrally buoyant detergents, the sole contribution of protein to the s value is observed. Previous studies on membrane proteins have implemented density matching of neutrally buoyant detergents with the aid of D₂O (Fleming, 2005; Reynolds and Tanford, 1976; Tanford et al., 1974). The use of D₂O to alter the solvent density has been used to effectively remove the slight detergent contributions by the neutral detergent to the protein-detergent complex sedimenting, if any. Therefore, to remove the detergent contribution that might be present, gravitationally transparent detergents may be used to determine the membrane proteins' size and distribution irrespectively of the bound detergent.

4.B. Methods

4.B.1. Sedimentation Velocity Experiments

In my AUC experiments, MerT/C₈E₄ complexes were monitored. MerT purified via Sepharose -Q IEC was concentrated to 0.2 ml or 1.0 ml and injected into an S-200 gel
filtration column equilibrated with 20 mM Tris pH 7.5, 100 mM NaCl, 0.25 mM TCEP, and 20 mM C₈E₄ buffer. Eluent fractions of 700 μL aliquots were collected and measured their concentration by absorbance at 280 nm. AUC samples consisted of 400 μL aliquots from the maximum gel filtration peaks, along with a 1:2 and 1:3 dilutions with buffer without detergent. While using C₈E₄, MerT/C₈E₄ micelle complexes contained additional dilutions with a final 12% D₂O (v/v) concentration that matched the micelle density of the detergent alone.

All experiments were performed on a Beckman Coulter XL-1 analytical ultracentrifuge at 20°C and 50,000rpm. Samples were loaded into aluminum cells having 1.2 cm long Epon double-sector optical centerpieces and sapphire windows. Cells were filled with 410 μL solvent as a reference and 400 μl of sample. Sedimentation profiles were acquired using absorbance 280 nm.

4.B.2. Numerical values for Sedimentation Velocity Analysis

Sedimentation velocity experiments involving detergent-proteins complexes were analyzed using the program SEDFIT version 12.1, (Gabrielson et al., 2007). In addition, theoretical solvent density and viscosity values were obtained from the program Sednterp (Hayes et al., 1995). Further, a 1.25 frictional ratio (f/f₀), corresponding to globular, hydrated particle was maintained for all runs (Salvay et al. 2007). Additionally, was adjusted in increments of 0.01 ml/g to obtain a Mᵦ of ~13 kDa. All c(s) distributions were calculated with a confidence level of 0.95.
4.C. Results for Sedimentation Velocity

In SV experiments with MerT/C₈E₄ complexes, two major and distinct sedimenting species were observed. The neutrally buoyant detergent gave rise to two species; the first having $S_{20w,\text{avg}}$ of 0.89S while the second species had a $S_{20w,\text{avg}} = 1.62S$. The RMS deviation for the fitting of all the sedimentation distributions was below 0.006. In addition, an adjustment of the provided $M_W = \sim 12.8$ kDa, which is within 2% of the expected 13 kDa molecular mass for the MerT monomer for species sedimenting around 0.89S. The second major species that sedimented was around the expected $M_W$ of dimer around 31.7 kDa, which is about 18% from the expected molecular mass of dimer, 26 kDa. And lastly, there appeared to be the formation of a minute third species around 2.6S, a possible tetrameric species with a $M_{W,\text{avg}}$ of 62.5 kDa, which is $\sim 17\%$ from a MerT tetramer of 56 kDa. There appeared to be a transition from the monomeric to dimer and, possible, tetrameric species with the change in detergent concentration. Specifically, C₈E₄ at 20mM demonstrated a relatively higher distribution of the monomer to the dimer species which is reduced upon dilution of the MerT/C₈E₄ complex at and below the CMC of C₈E₄, 6.5 mM and 5 mM respectively. In addition, the formation of the possible tetramer became more apparent with dilution of the MerT/C₈E₄ complex which is more clearly seen in the sedimentation distributions with D₂O. The effect of D₂O on the sample did not change the relative distributions of the monomer but had more $s$ values that were closer to one another than the samples without D₂O.
4.D. Discussion of SV experiments

Analytical ultracentrifugation was used to determine the oligomeric state of the mercury transporter, MerT, solubilized in C₈E₄. MerT/C₈E₄ complexes revealed two major oligomeric species that of a monomer and a dimer; also, a third possible species that hinted to a tetramer.

MerT/C₈E₄ complexes indicated that MerT multimer formation could appear as two major species, a monomer and a dimer. There was a fluctuation of the monomeric to the dimer MerT observed when MerT was in the C₈E₄ detergent. More specifically there seems to be a higher monomer formation versus the dimer when the detergent solution is above the CMC of C₈E₄. Yet dimer formation is present prior to the dilution of MerT/C₈E₄ complex, indicating that the active form of MerT might be a dimer. Upon lowering the C₈E₄ detergent, the distribution of monomer to dimer changes such that there is a near-even distribution of each species, with a monomer species still present in higher abundance. The relative distribution of monomers and dimers are retained at and below the CMC of C₈E₄ suggesting equilibrium between monomers and dimers of MerT. Correspondingly, this multimeric association is supported via cross-linking experiments performed with 8His-MerT. On another note, the juxtaposition of SEC and AUC data, like Rₛ and Mₘ, were not compared since the MerT/C₈E₄ complex in SEC takes into account the detergent contribution whereas in AUC the purpose of using C₈E₄ was to remove detergent contribution only observing the MerT oligomers.
Conclusions

From the above experiments conducted, the MerT oligomerization in non-ionic detergents is that of a dimer. In chemical cross-linking experiments, the formation of dimers was demonstrated with the addition of the thiol reactive cross-linker, BM(PEG)$_2$. Although, higher order oligomers of trimers and tetramers were observed with the use of BM(PEG)$_2$, their presence was not observed in using the amine reactive cross-linker BS$_3$. However, the addition of BS$_3$, again, supported the formation of dimers by shifts in SDS-PAGE gels. In addition, the use of SEC gave a range of multimers in three different non-ionic detergents from a hexamer to a dimer. Yet, only two detergents maintained monodisperse distribution of MerT/detergent complexes, C$_8$E$_4$ and DDM. Yet, the unique characteristic of C$_8$E$_4$ as a neutrally buoyant detergent, or gravitationally transparent detergent, allowed a direct determination of the MerT oligomeric species present. More specifically, MerT exists as monomers and dimers in non-ionic detergents and the formation of dimers is increased in C$_8$E$_4$ detergent concentrations below the CMC. The formation of dimers at concentration below the CMC suggests that dimer oligomerization might be the natural occurring form of the mercury transporter.

To absolutely determine the functional organization of MerT it must be determined by X-ray crystallography. More specifically, here it was determined that the protein maintained dimeric forms in the detergents implemented. Furthermore, the MerT/detergent could be lowered to values near the CMC, for DDM and C$_8$E$_4$, while maintaining stable complexes. Currently, crystal screenings MerT/OG complexes are underway.
Figure 5.1. The density matching method to effectively remove the detergent contribution to the sedimenting particle in analytical ultracentrifugation. A. The buoyant molecular weight observed in non-density matched conditions such that the detergent associated contributes to the sedimenting particle size. B. The density matched condition where the density of the solution has been matched to that of the detergents effectively removing the detergent contribution and observing solely the protein contribution to the buoyant particle sedimenting.
Figure 5.2. Sedimentation Distribution of MerT-C$_8$E$_4$ complex. Two distinct species are observed, one ~1S and another ~1.5S. A possible third sedimenting particles ~2.1S.

Table 5.1. SV values of parameters obtained from MerT-C$_8$E$_4$ complex

<table>
<thead>
<tr>
<th>[C8E4]</th>
<th>$S_{20w,1}$</th>
<th>$S_{20w,2}$</th>
<th>Monomer $M_w$, kDa</th>
<th>Dimer $M_w$, kDa</th>
<th>RMSD</th>
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<tr>
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<td>6.5mM</td>
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<td><strong>12.8</strong></td>
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<td>5mM</td>
<td><strong>1.018</strong></td>
<td>1.734</td>
<td><strong>12.8</strong></td>
<td>28.6</td>
<td>0.00264</td>
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Figure 5.3. Sedimentation Distribution of MerT-C₈E₄ in 12% D₂O. SV distribution resembled that without D₂O, yet profiles demonstrated more uniformity in S-values obtained. Particle sedimenting ~2.1S was not observed at 20mM yet was observed at lower C₈E₄ concentrations.

Table 5.2. SV values of parameters obtained from MerT-C₈E₄ complex in 12% (v/v) D₂O.

<table>
<thead>
<tr>
<th>[C8E4]</th>
<th>S₂₀w,₁</th>
<th>S₂₀w,₂</th>
<th>Mᵦ₁, kDa</th>
<th>Mᵦ₂, kDa</th>
<th>RMSD</th>
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<tr>
<td>20mM</td>
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<td>1.602</td>
<td>12.6</td>
<td>33.8</td>
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<tr>
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<td>1.668</td>
<td>12.8</td>
<td>29.5</td>
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References


