CHAPTER 2

Preparation of OmpA proteins and phospholipid vesicles

Acknowledgement

Experiments were done in collaboration with Dr. Judy E. Kim. Dr. Kim initially expressed and purified wild-type, W7, W15, W57, W102, and W143 OmpA proteins. The protocols from these experiments are included as a reference for future students and postdoctoral scholars studying OmpA.

2.1 INTRODUCTION

β-barrel membrane proteins

There are two structural motifs of integral membrane protein, α-helical bundles and β-barrels (Bowie, 2005). A two stage folding model has been proposed for the α-helical bundles where each helix inserts into the membrane independent of the others, followed by lateral association of the helices to form the native structure (Figure 2.1) (Popot & Engelman, 1990). This model was indeed supported from studies of bacteriorhodopsin fragments and from helical segments of the shaker K^+ channel (Kahn & Engelman, 1992; PeledZehavi et al., 1996; Hunt et al., 1997). This two-stage mechanism reasonably explains the folding of those membrane proteins with hydrophobic transmembrane domains but would not explain the folding for those with polar side chains in their transmembrane helices, including those in ion channels.

β-barrel membrane proteins are expected to fold differently from the α-helical bundles because their residues alternate between hydrophobic and hydrophilic states. The outer surface contains hydrophobic residues for interaction with the cell membrane while the inner surface is mostly hydrophilic to allow molecules to traverse the pore. Also, the two-stage model would not be reasonable since many backbone hydrogen bonds would be broken if individual β-sheets insert as autonomous folding units. The average hydrophobicity of β-barrels is low and the design of the β-barrels is suited for spontaneous insertion since each transmembrane (TM) segment is not very hydrophobic (Vogel & Jahnig, 1986). The current folding model for β-barrels is concerted folding followed by insertion into the bilayer (Figure 2.1).

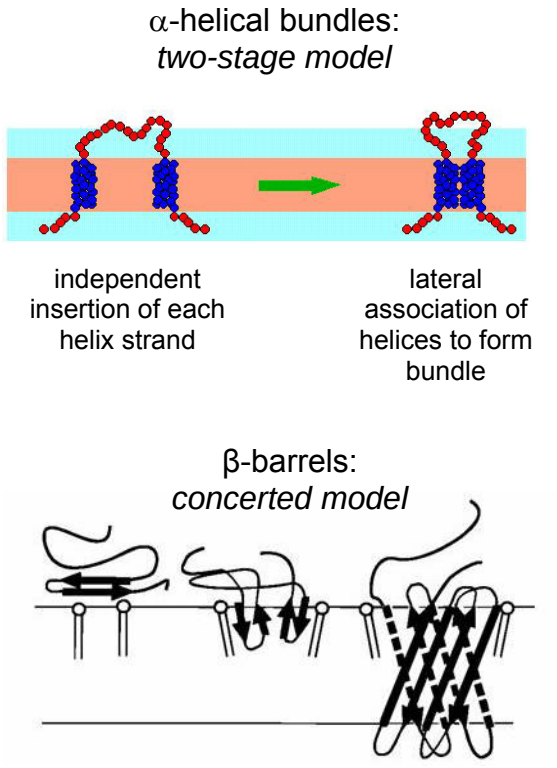

Figure 2.1. Schematic of the two-stage model proposed for general folding of α-helical bundles (top) and the concerted model for the folding of β-barrels (bottom) into the lipid bilayer.

All known integral membrane proteins with transmembrane β-sheet secondary structure form barrels with at least eight strands connected by hydrogen bonds and long-range interactions (Schulz, 2002). β-barrel membrane proteins exist in the outer membrane of Gram-negative bacteria, chloroplasts, and mitochondria. Gram-negative bacteria lack a source of energy, namely ATP, in the periplasm, so β-barrel membrane proteins must devise a way to fold spontaneously.

β-barrel membrane proteins perform diverse functional roles such as bacterial adhesion, phospholipase and protease activity, selective and voltage-gated intake of sugars and ions, ligand-gated uptake of iron siderophore complexes, and protein or drug export. However, because β-barrel membrane proteins are less abundant than α-helical proteins, they have not received as much attention (Bigelow et al., 2004).

The outer membrane

Gram-negative bacteria, such as *E.coli*, contain a cell envelope composed of two membranes, the outer and inner membranes (Figure 2.2). The aqueous periplasm separates the two membranes and contains the peptidoglycan layer, a major cell wall component. The inner and outer membranes are different in structure, composition, and function. The outer membrane (OM) is highly asymmetric. The inner leaflet is made up of phospholipids and the outer leaflet is composed of lipopolysaccharide (LPS), which is crucial to the barrier functions of the OM. Half of the OM is composed of two types of proteins, lipoproteins and integral outer membrane proteins (Omps). Most of these Omps are specifically porins and outer membrane protein A (OmpA). The Omps span the OM with amphipathic, antiparallel β-strands that form a barrel structure, allowing Omps to serve as channels into the cell. This function is essential to bacterial survival as it allows

the OM to act as a barrier, allowing ions and molecules to be imported to and exported from the cell as well as preventing entry of toxins. For example, the *E. coli* OM is impermeable to bile salts, which allows this bacterium to survive in intestines (Koebnik et al., 2000; Tamm et al., 2001).

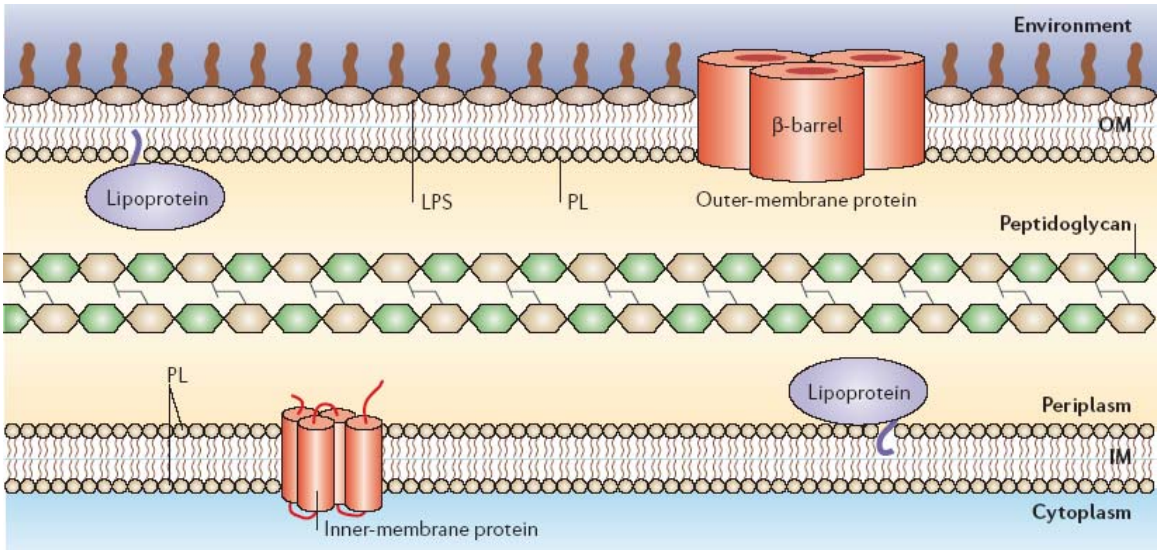

Figure 2.2. Schematic illustrating the general cell envelope of *E. coli*. The inner membrane (IM) is separated from the outer membrane (OM) by the aqueous periplasm, which contains the peptidoglycan cell wall component. Inner membrane proteins contain α-helical transmembrane domains while outer membrane proteins contain β-barrel transmembrane domains. Both the IM and OM contain lipoproteins that are attached to their periplasmic sides. Adapted from (Ruiz et al., 2006).

Biosynthesis and transport of outer membrane proteins

All bacterial proteins, including Omps, are synthesized in the cytoplasm with an N-terminal signal sequence that directs them to the SecY/E/G translocon, which then translocates Omps through the inner membrane (IM) into the periplasm. The signal sequence is cleaved off by signal peptidases at the outer leaflet of the IM. Insertion and folding of Omps into the OM is spontaneous (Tamm et al., 2001). There is debate about how OM components traverse the periplasm. Components may reach the OM through bridges linking the IM and OM or chaperones that escort them. It is suggested that chaperones and protein-folding factors help bind unfolded Omps to prevent aggregation and misfolding as they transport through the periplasm (Ruiz et al., 2006). Several periplasmic proteins have been discovered such as Skp, SurA, FkpA, and DegP, although their exact roles are not yet known (Duguay & Silhavy, 2004; Mogensen & Otzen, 2005). Skp has been suggested to bind Omps as they exit the Sec machinery to prevent aggregation in the periplasm (Chen & Henning, 1996; Schafer et al., 1999; Bulieris et al., 2003; Walton & Sousa, 2004). However, further studies on this topic are required as other parallel pathways for targeting Omps to the OM are possible (Rizzitello et al., 2001; Wu et al., 2005); (Ruiz et al., 2006).

Outer membrane protein A

Outer membrane protein A (OmpA) from *E. coli* is a monomeric β-barrel membrane protein with 325 residues (Figure 2.3). It is one of the major Omps of *E. coli* with ~100,000 copies/cell (Koebnik et al., 2000). Its main function is to maintain structural integrity of the cell surface. OmpA contains a transmembrane N-terminal domain and a water-soluble periplasmic C-terminal domain (Figure 2.4), which has been

proposed to interact with the peptidoglycan layer and provide a physical link between the outer membrane and the cell wall (Koebnik et al., 2000). It has been observed that a mutation in OmpA and another lipoprotein, Lpp, leads to spherical cells that cannot survive outside well-balanced osmotic conditions (Sonntag et al., 1978). OmpA is also important for bacterial conjugation (Ried & Henning, 1987), and can serve as receptors for phages and colicines (Chai & Foulds, 1974; Vanalphen et al., 1977; Sonntag et al., 1978; Wang, 2002). Additionally, OmpA forms ion channels in planar lipid bilayers (Sugawara & Nikaido, 1992; Sugawara & Nikaido, 1994; Arora et al., 2000), resulting in another topological model where a minor conformation of OmpA resembles that of a 16-stranded β-barrel porin (Stathopoulos, 1996; Zakharian & Reusch, 2005). Since this protein is so abundant in Gram-negative bacteria, it is an important target in defense against bacterial pathogens (Weiser & Gotschlich, 1991; Prasadarao et al., 1996; Belaaouaj et al., 2000; Soulas et al., 2000).

There are high-resolution structures of the transmembrane domain, which were obtained from X-ray crystallography (Pautsch & Schulz, 1998; Pautsch & Schulz, 2000) and solution NMR spectroscopy (Arora et al., 2001) (Figure 2.4). Furthermore, NMR dynamics reveal that the extracellular loops of OmpA are highly dynamic and possibly explain the ability of OmpA to function as a membrane channel. The flexible ends contain the aromatic and polar residues, which make contact with the polar head groups of the lipids or detergents (Arora et al., 2001). There is currently no structure of the C-terminal domain. A network of salt bridges and hydrogen bonds was observed within the pore and may explain high thermal stability of OmpA. It has been debated whether OmpA can act as a pore since no continuous transmembrane channel was observed in the

crystal structure. OmpA also has a high tolerance against mutational alterations to its shape, especially in the loop, turn and lipid bilayer facing regions of the barrel, thus changes in structure can be applied without affecting the sturdy β-barrel (Freudl, 1989; Ried et al., 1994; Koebnik & Kramer, 1995). Molecular dynamic simulations of the interactions of the transmembrane region with detergents as well as the self-assembly process of OmpA have been reported (Domene et al., 2003; Bond et al., 2006; Bond & Sansom, 2006).

OmpA is used as a well-suited model for studying the folding and insertion of β-barrel membrane proteins. Few integral membrane proteins were shown to fold *in vitro* from a fully denatured state (Booth et al., 2001). It was first shown by Surrey and Jahnig that OmpA spontaneously inserts and folds into phospholipid bilayers from a completely unfolded and soluble state in 8 M urea (Schweizer et al., 1978), without the use of detergents (Surrey & Jahnig, 1992). The moderate hydrophobicity of the β-sheet structure makes this observation possible. Refolding is initiated by dilution of the denaturant into a solution of either detergent micelles or small unilamellar vesicles (SUVs) and, depending on lipid properties, large unilamellar vesicles (LUVs). Folding studies with detergents add complexity to the measurements by complicating spectra and additional experimental work must be done to remove the detergents once the protein has been solubilized. The absence of detergent solubilization makes OmpA an ideal representative for studying the folding mechanism of monomeric β-barrels.

Previous studies of OmpA folding have used a range of different phospholipids and detergents at varying concentrations (Kleinschmidt et al., 1999). OmpA was reported to fold into 64 different detergents and phospholipids containing different head

groups, no net charge, and a hydrocarbon chain of 7 to 14 carbon atoms. Folding of OmpA has been monitored by SDS-PAGE because of the differing electrophoretic mobilities of folded and unfolded OmpA when samples are not boiled. Folded OmpA is more compact and migrates at 30 kDa while unfolded OmpA migrates at 35 kDa (Schweizer et al., 1978). Phage inactivation (Schweizer et al., 1978), single-channel conductivity measurements (Stathopoulos, 1996; Arora et al., 2000), CD, Raman, and FT-IR spectroscopies (Dornmair 1990, Surrey 1992, 1995, Kleinschmidt 1999, Rodionova 1995, Vogel and Jahnig 1986) have shown that the 30 kDa form is the folded and functionally active OmpA.

OmpA refolds into the lipid bilayer with its C-terminal domain located at the exterior of the vesicle (Figure 2.5) (Surrey & Jahnig, 1992). The folding efficiency and kinetics into the bilayer are dependent on factors such as temperature (Kleinschmidt & Tamm, 1996; Kleinschmidt et al., 1999), pH (Doring et al., 1995), and lipid bilayer composition and properties (Doring et al., 1995; Kleinschmidt & Tamm, 2002). OmpA refolds into detergents only in the micellar form. The arrangement of micelles around OmpA is thought to be a monolayer or ellipsoid arrangement (Kleinschmidt et al., 1999). Folded OmpA is protected from trypsin digestion by the lipid bilayer, similar to native OmpA in the *E.coli* outer membrane. Trypsin digestion of the folded form produces a 24 kDa fragment, while the unfolded 35 kDa form is completely degraded by trypsin (Schweizer et al., 1978).

OmpA contains 5 native tryptophans (Trp) that reside within the transmembrane domain at residue positions 7, 15, 57, 102, and 143 (Figure 2.5). W102 is the only Trp residue that faces the interior of the pore while the other 4 Trp face toward the bilayer

environment (Figure 2.6). Investigations of refolding kinetics have been performed on OmpA single tryptophan mutants, where 4 of 5 Trp are mutated to phenylalanines (Kleinschmidt et al., 1999). Fluorescence of these native Trp residues are central to the work described in this thesis. The following chapters will describe the applications of fluorescence spectroscopy in investigations of the microenvironment of single Trp mutants. In addition to these five full-length Trp mutants, we expressed the 176 amino acid truncated proteins by removal of the periplasmic C-terminal domain. The purpose of these truncated mutants is to determine the effects of the C-terminal tail on the refolding process and the transmembrane structure. A total of 10 mutants composed of 5 full-length and 5 truncated (t) Trp mutants have been expressed and purified for studies: W7, W7t, W15, W15t, W57, W57t, W102, W102t, W143, and W143t.

To investigate refolding using fluorescence energy transfer kinetics, the following mutants were prepared: W7/C290S/C302S/A175C and W7t/A175C (truncated variant).

OmpA primary sequence

(MKKTAIAIAVALAGFATVAQA)APKDNTWYTGAKLGWSQYHDTGFINNNGPTHENQLGAGAFG
GYQVNPYVGFEMGYDWLGRMPYKGSVENGAYKAQGVQLTAKLGYPITDDLDIYTRLGGMVWR
ADTKSNVYGKNHDTGVSPVFAGGVEYAITPEIATRLEYQWTNNIGDAHTIGTRPDNGMLSLGVSY
RFGQGEAAPVVAPAPAPAPEVQTKHFTLKSDVLFNFNKATLKPEGQAALDQLYSQLSNLDPKDGS
VVVLGYTDRIGSDAYNQGLSERRAQSVVDYLISKGIPADKISARGMGESNPVTGNTCDNVKQRAA
LIDCLAPDRRVEIEVKGIKDVVTQPQA

Figure 2.3. The amino acid sequence of wt-OmpA is shown. The signal sequence prior to the OmpA sequence is shown in parentheses. Native Trp (W7, W15, W57, W102, W143) are highlighted in blue. The last residue in all truncated proteins is highlighted in red. A175 is shown in green and C290 and C302 that were mutated to serines are shown in orange.

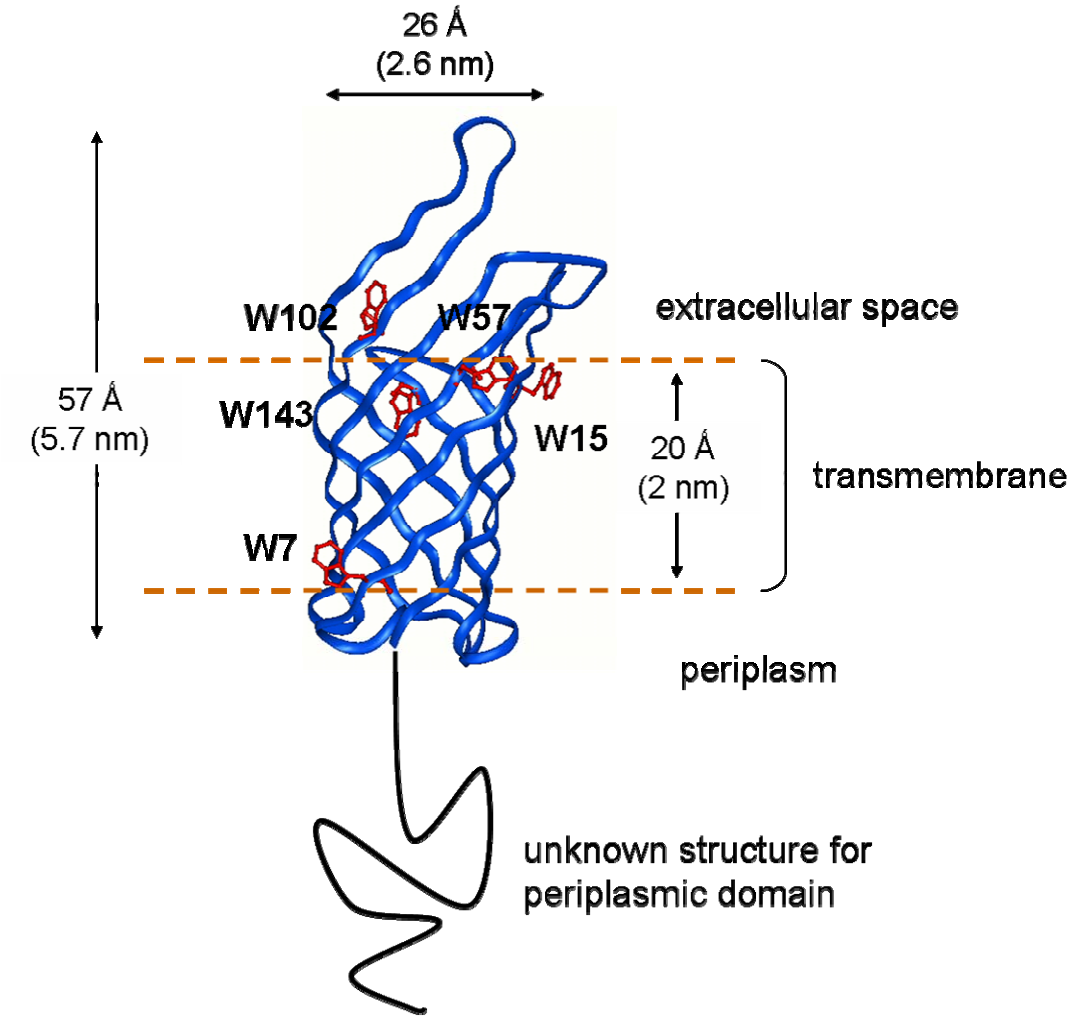

Figure 2.4. Structure of the OmpA transmembrane domain (residues 1-171), solved by X-ray crystallography (Pautsch & Schulz, 1998). The 5 native Trp are shown along with the approximate dimensions and an illustration of the C-terminal tail in the periplasmic space. The approximate location of the transmembrane region is shown with the red, dashed lines.

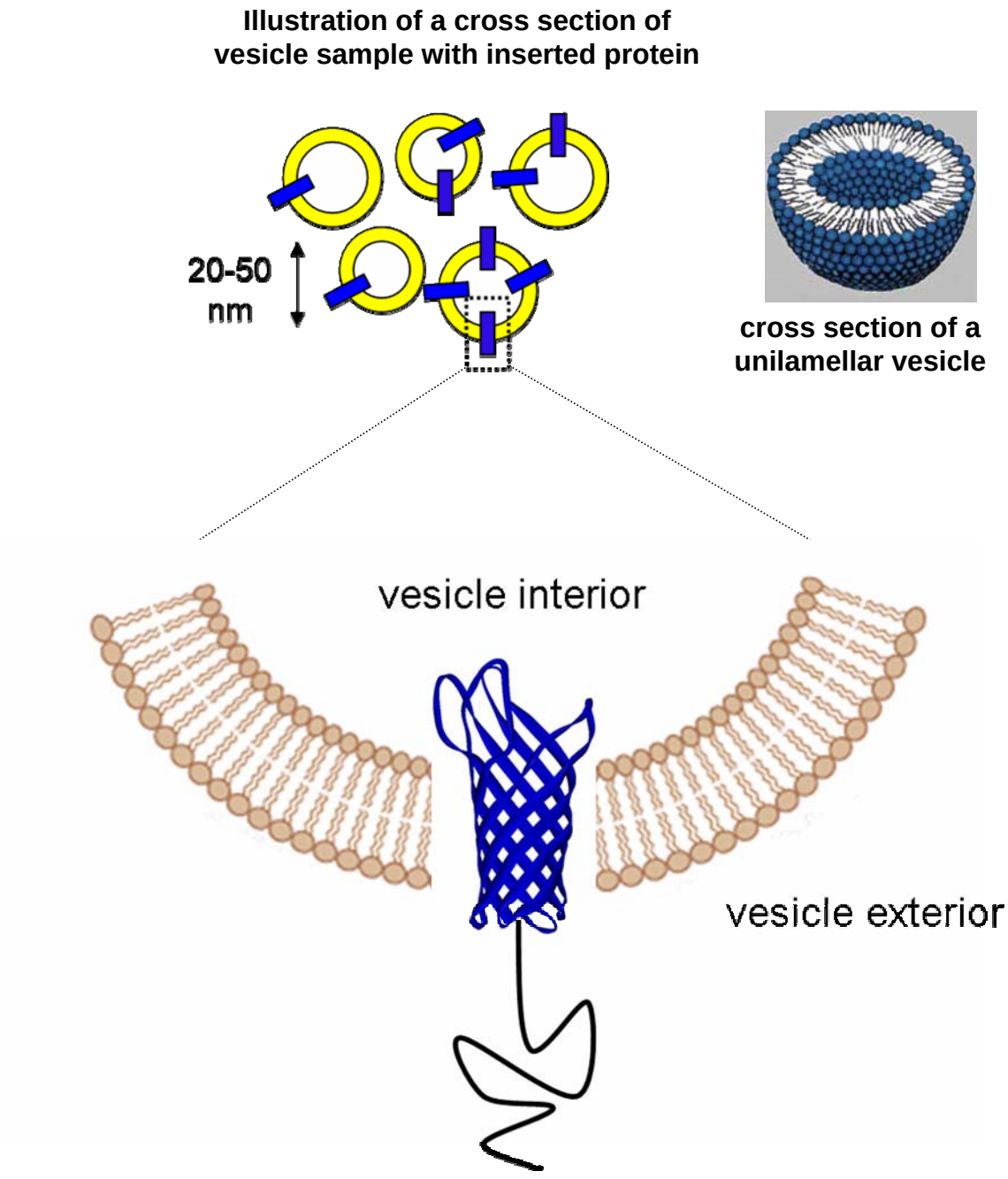

Figure 2.5. Schematic of the oriented insertion of OmpA into the lipid vesicles in refolding *in vitro* experiments. The C-terminus tail of OmpA is located on the exterior of the vesicle while the extracellular loops are on the interior of the vesicle.

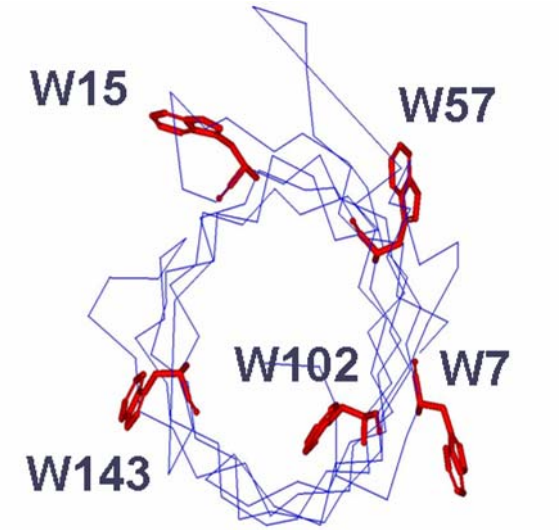

Figure 2.6. Cross section of the OmpA transmembrane structure displaying the Trp orientations. W102 is the only Trp that faces the OmpA pore. The other 4 Trp face the exterior of the bilayer and interact with the lipid bilayer.

2.2 MATERIALS AND METHODS

Preparation of small unilamellar vesicles (SUVs)

A 20 mg/ml solution of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) in chloroform (Avanti Polar Lipids) was divided into 1 mL aliquots. A glass buret was used to transfer the solution to small glass vials. Plastic pipette tips are not recommended since the lipids are in chloroform. By dividing the solution up into batches, we minimized the variations in lipid concentration since chloroform evaporates over time, even when parafilmed. The 1ml lipid solutions were stored in the -20^{o}C freezer. Lipids used for experiments were all under 6 months old and were not used once this time period has passed. Brominated lipids can be used up to one year.

The protocol for SUV preparation is adapted from the literature (Surrey & Jahnig, 1992) . Typically, the 1 mL aliquots were dried in a glass vial under a stream of argon followed by vacuum for a few hours. For brominated lipid experiments, we used 1-palmitoyl-2-stearoyl(6-7 or 11-12) dibromo-sn-glycero-3-phosphocholine (6,7-DiBr or 11,12-DiBr) (Avanti Polar Lipids). To prepare brominated vesicles, a 1:3 molar ratio of 6,7- or 11,12-DiBr:DMPC solution was made by adding the appropriate amount of 25 mg/mL 6,7-DiBr or 11,12-DiBr solution to DMPC prior to drying under argon and vacuum.

The dried lipids were hydrated in 20 mM KPi buffer, pH 7.3, to a lipid concentration of 5 mg/mL using a bath sonicator to loosen and disperse the lipid film in the glass vials. To produce the SUVs, the lipid solution was ultrasonicated for 30 minutes using an ultrasonicator microtip (Branson) at 50 % duty cycle and 200-300 W in a room temperature water bath. Particulates such as titanium dust from the sonicator

were removed either by centrifugation or by filtering the solution through a 0.22 μm filter. The SUVs were added directly into a solution of 20 mM KPi, pH 7.3, which had been equilibrating in the 35°C oven, producing a final lipid concentration of 1 mg/mL, and then equilibrated overnight at 35°C. SUVs were used the day after preparation for all experiments. Prior to protein injection, vesicles were either kept in a 35°C oven, on ice, or in the cold room (4°C).

Characterization of SUVs by dynamic light scattering

Vesicles were characterized on the day of use by dynamic light scattering (Precision Detectors, Inc) at 3 detector angles, 45°, 90°, and 135°. The 1 mg/mL (1.5 mM) vesicle solution was filtered into a small glass test tube that fit into the sample holder. Occasionally, the vesicles had to be further diluted with phosphate buffer due to larger particulates that did not filter out or due to samples being too concentrated, resulting in a measured intensity that shuts off the detector. Measurements were taken at 30°C using a viscosity value of 0.00798 and refractive index of 1.33. Accumulations of 60 acceptable scans were recorded 4-10 times for each sample using the Precision Deconvolve program. Aperture size and laser power were varied until the intensity counts reached about 1-1.5 million. Auto-correlation functions in the Precision Elucidate program produced the hydrodynamic radius.

Site-directed mutagenesis to produce full-length, single Trp OmpA plasmids

The work in this section to produce the full-length single tryptophan mutants was completed by Dr. Judy E. Kim. The plasmid pET1102, which contains the gene for full-length (325 residue) OmpA with a single tryptophan at position 7 (W7), was a gift from Professor Lukas K. Tamm at the University of Virginia (Kleinschmidt et al., 1999).

Several rounds of site-directed mutagenesis using Quik-change (Stratagene) produced the other 4 full-length, single tryptophan OmpA mutants. Initially, pET1102 was modified to encode for a tryptophan-less OmpA mutant, W0, in which all five of the native tryptophans are replaced by phenylalanines. Mutation of these non-native phenylalanines (phe) to trp then produced the remaining four plasmids for single tryptophan OmpA: W15, W57, W102, and W143.

Site-directed mutagenesis to produce truncated, single Trp OmpA plasmids

The 147-residue C-terminal domain for each of the five single tryptophan mutants was removed via a stop codon at position 177 using site-directed mutagenesis. These plasmids produce the five truncated (176-residue) single-tryptophan OmpA mutants: W7t, W15t, W57t, W102t and W143t. The PCR products were transformed into XL1-Blue supercompetent cells. Cells were picked and grown overnight in 5ml cultures. DNA was extracted using the Qiagen Miniprep kit and submitted to the Caltech Sequence and Structure Analysis Facility for DNA sequence verification.

Expression and extraction of OmpA

OmpA proteins were extracted from the outer membranes and purification protocols were adapted from published protocols (Teather et al., 1980; Surrey & Jahnig, 1992; Surrey et al., 1996). The OmpC-free *E. coli* strain JF701 (*E. coli* Genetic Stock Center, Yale University) was used to obtain wild-type OmpA. JF701 cells were grown overnight at 37^{o}C in 50 mL of sterile 1% bactotryptone, 0.5% yeast extract media supplemented with 25 μg/ml streptomycin. Cells were pelleted by centrifugation (~6000 rpm), washed with sterile media, and pelleted again. The cells were gently and quickly resuspended with sterile media and transferred to 6 L of the fresh, sterile media. Cells

were grown at ~35-37^{o}C with shaking and were harvested at OD_{600} = 0.9 with centrifugation. Cell pellets were washed twice with 10 mM Tris-Cl, pH 7.8.

The OmpA- and OmpF-free *E. coli* strain JF733 (*E. coli* Genetic Stock Center, Yale University) were used to overexpress OmpA Trp mutants via heat-shock transformation of the mutant plasmids, similar to procedures from the Quik-change kit. Glycerol stocks of the cells were prepared and stored in the -80 ^{o}C freezer. Stabs of the glycerol stocks were then used to inoculate 300 ml of LB media containing 0.5% glucose and 50 μg/ml of ampicillin and were grown at 37^{o}C with shaking overnight (~10-12 hrs). Cells were washed the next morning with sterile LB and then transferred to 6 L of fresh sterile LB containing 50 μg/mL ampicillin. OmpA expression was induced with 1 to 1.5 mM isopropyl β-D-thiogalactoside (IPTG) at an OD_{600} = 0.6. Cells were harvested at OD_{600}=1.3 via centrifugation and washed twice with 10 mM Tris-Cl, pH 7.8.

OmpA extraction procedures may be continued from this point, or the extraction may be continued at a later time. In this case, cell pellets were stored at either -20 ^{o}C or -80 ^{o}C until ready to extract, upon which the cell pellets were defrosted in a room temperature water bath.

Defrosted cell pellets were resuspended in 50 mL of cold 0.75 M sucrose, 10mM Tris-HCl, pH 7.8, followed by slow addition of 50 ml of cold 20 mM EDTA, 0.5 mg/mL lysozyme over a span of 1 minute. The cell suspension was stirred in an ice bath with periodic smashing of the cells with a spatula until cell clumps were minimal or not visible. A solution of PMSF in isopropanol was added to the solution to a final concentration of 1 mM. The solution was then sonicated on ice with a standard tip horn for 5 minutes at 50% duty cycle. The lysed cells were then centrifuged at 1500 x g for 15

minutes to remove unlysed cells and other cellular matter such as spheroplasts (cell with no cell wall). The supernatant was spun for 90 minutes at 150,000 x g to pellet the cell membranes. The red-brown pellets containing OmpA in cell membranes were separated from the supernatant containing soluble proteins and resuspended in 70 mL of pre-extraction buffer, composed of 3.5 M urea, 20 mM Tris-HCl, pH 9, 0.05% 2-mercaptoethanol, and stirred in a 50^{o}C water bath. The resuspended solution was ultracentrifuged at 150,000 x g for 2 hours. The resulting supernatant contains peripheral membrane proteins and the pellet contains cell membranes and OmpA. The pellets were resuspended in a buffer made of a 1:1 mixture of isopropanol:extraction buffer (8 M urea/20 mM Tris-HCl, pH 8.5/0.1% 2-mercaptoethanol) with stirring at 50 ^{o}C until no clumps were visible. Ultracentrifugation at 150,000 x g for 90 minutes separated the OmpA from the cellular membrane. An alternative is preparative centrifugation at 40,000 x g for 90 minutes, which was also fast enough to separate OmpA. The supernatant, containing OmpA protein, was then stored at 4^{o}C until further purification.

Purification of wild-type OmpA and Trp mutants

Anion-exchange chromatography using a Q Sepharose Fast Flow column (Pharmacia) equilibrated with a solution of 8 M urea, 0.5% 2-mercaptoethanol, 15 mM Tris, pH 8.5 (buffer A) was used to purify crude OmpA. OmpA was eluted from the column using a linear NaCl gradient (0-200 mM). Buffer B contained Buffer A plus 200 mM NaCl. Fractions that contained OmpA were combined as determined using UV-visible absorption and SDS-PAGE. Purified OmpA was concentrated by ultrafiltration (Amicon, PM-10 for full-length mutants or YM-3 for truncated mutants) and washed several times to exchange buffer into 8 M urea, 20 mM potassium phosphate, pH 7.3. It

is recommended that some DTT be added to the storage buffer to prevent oxidation of cysteine residues. Concentrated protein stocks were aliquoted into 1.5 mL tubes and stored at -80 ^{o}C or -20 ^{o}C.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to published protocols (Laemmli, 1970) using the PhastSystem gel electrophoresis (Pharmacia), which contains both a separation and a development unit. This instrument is more convenient compared to traditional SDS-PAGE setups because pre-cast polyacrylamide gels and SDS buffer strips are used. High-resolution separation is obtained using a thermostatic plate that maintains accurate temperatures during the separation. SDS-PAGE was performed with unheated protein samples mixed with reducing staining buffer. Polyacrylamide gels (12.5%) were run at 15 ^{o}C to prevent overheating. Coomassie blue or silver staining methods were used to visualize protein bands.

Site-directed mutagenesis to produce W7/C290S/C302S, W7/A175C, and W7t/A175C

To produce the single cysteine variant of W7, two rounds of site-directed mutagenesis were used to mutate the two native cysteines in the C-terminal tail to serines. This plasmid was expressed, purified, and used as a control protein during the dansyl-labeling reaction of W7/A175C. A third round of mutagenesis was used to mutate the double cysteine mutant from A175 to cysteine. The single cysteine variant of W7t is a bit more straightforward since it lacks cysteine residues. A single round of site-directed mutagenesis was used to produce the A175C. Correct gene sequence was verified by DNA sequencing.

Mass spectrometry

Protein in urea was placed in 50% acetonitrile, 0.1% trifluoroacetic acid using the ZipTip pipette tips (Millipore). Electrospray mass spectrometry was performed by the Protein/peptide Analytical Lab.

Protein reconstitution

Refolding of OmpA proteins into detergent micelles or SUVS was performed using published procedures (Surrey & Jahnig, 1992). Detergent micelles were prepared from octyl-β-D-glucopyranoside (OG) to a final concentration of 10 mg/mL in 20mM KPi, pH 7.3. This concentration is ~1.4 times the critical micelle concentration (CMC), thus ensuring that the detergents exist in the micellar form. OmpA was refolded by a 20 fold dilution of urea into the OG micelles and allowed to fold for at least 30 minutes prior to data measurements. The OmpA to OG molar ratio was ~ 1:7500.

For vesicles, OmpA was diluted into 1 mg/mL DMPC vesicles to a final molar ratio of 1:300 and allowed to fold for at least ~ 3 hrs in a 35 ^{o}C oven before spectroscopic measurements. To show differential gel electrophoretic mobility, SDS-PAGE was used to confirm folding of the protein, since folded protein migrates at 30 kDa and unfolded protein migrates at 35 kDa.

2.3 RESULTS AND DISCUSSION

Choice of phospholipids and characterization of SUVs

DMPC vesicles were chosen to initially study refolding of OmpA since these particular phospholipids were originally used by Surrey and Jahnig (1992). We attempted to fold OmpA into DOPC vesicles since the Tamm laboratory used this

particular lipid (Kleinschmidt & Tamm, 1996). However, we observed that the emission maxima of proteins in DOPC were not as blue-shifted as those in DMPC. Gels also revealed that about twice as much OmpA was folded for proteins in DMPC compared to DOPC. Based on these results, we decided to continue our studies using DMPC. Inefficient folding into DOPC may be due to its larger hydrophobic thickness of 27 Å compared to the 23 Å thickness of DMPC (Lewis & Engelman, 1983). It had been determined that the thickness of the hydrocarbon core affects the folding of OmpA. More efficient folding is observed when there is a better match between the hydrocarbon thickness and the transmembrane region (Kleinschmidt & Tamm, 2002).

Previous studies reported that for DMPC lipids, OmpA will only fold when in the SUV form and not the large unilamellar vesicle form (>100 nm diameter) (Surrey & Jahnig, 1992). Lipids of 14 carbon chains or more in the SUV form are more amenable to OmpA folding due to the high surface curvature and defects, exposing more of the interior of the hydrophobic chain, which could serve as nucleation sites for folding. However, if OG detergent was added to DMPC LUVs, OmpA was permitted to spontaneously fold.

DLS data is shown in Figure 2.7. DMPC vesicle size distributions are ~ 20-50 nm in diameter and do not vary drastically between different vesicle preparations. These sizes are close to those reported for DMPC SUVs (Surrey & Jahnig, 1992).

Protein expression, purification, reconstitution

Table 2.1 lists all the mutants prepared, along with their residue length, molecular weights, and extinction coefficients. It was previously shown that OmpA is soluble in aqueous urea (Schweizer et al., 1978) due to its low hydrophobicity. Once OmpA is

incorporated into the cell membrane, it could not be solubilized by simple addition of urea. This allowed the peripheral membrane proteins to be removed. Figure 2.8 summarizes the extraction procedure. Despite the lower urea concentration of 3.5 M to remove peripheral proteins, SDS-PAGE of peripheral proteins sometimes revealed a small band corresponding to OmpA. However, yields of crude OmpA were high enough that purification from peripheral proteins was not necessary. Yields of purified OmpA varied from 3 to 5 mg/L culture. During purification, OmpA elutes starting at Buffer B percentage of ~10-15 %. Rather than a sharp peak, the protein elutes over a broad range since it is purified in the unfolded form (Figure 2.9).

Unfolded OmpA migrates on the gels with an apparent molecular mass of 35 kDa while folded OmpA in micelles and vesicles migrates at 30kDa (Figure 2.10). Samples of folded protein were not heated prior to SDS-PAGE so that that the folded state of the protein could be detected on the gel. SDS-PAGE of FPLC-purified protein showed a purity of ~90% (Figure 2.10). For the full-length protein, a faint band at 24 kDa was always observed and presumed to be some digested protein since this corresponded to the size of the tryptsin digested fragment. Although PMSF was added to the extraction procedures, it did not seem to significantly reduce this lower molecular weight band.

Mass spectrometry

Membrane proteins typically do not fly well during mass spec analysis. Electrospray mass spec of the proteins revealed molecular masses that are close to the calculated mass but were always ~100-300 kDa heavier than the calculated mass. Furthermore, the mass peak was always broad and contained multiple peaks that are separated by ~20 amu.

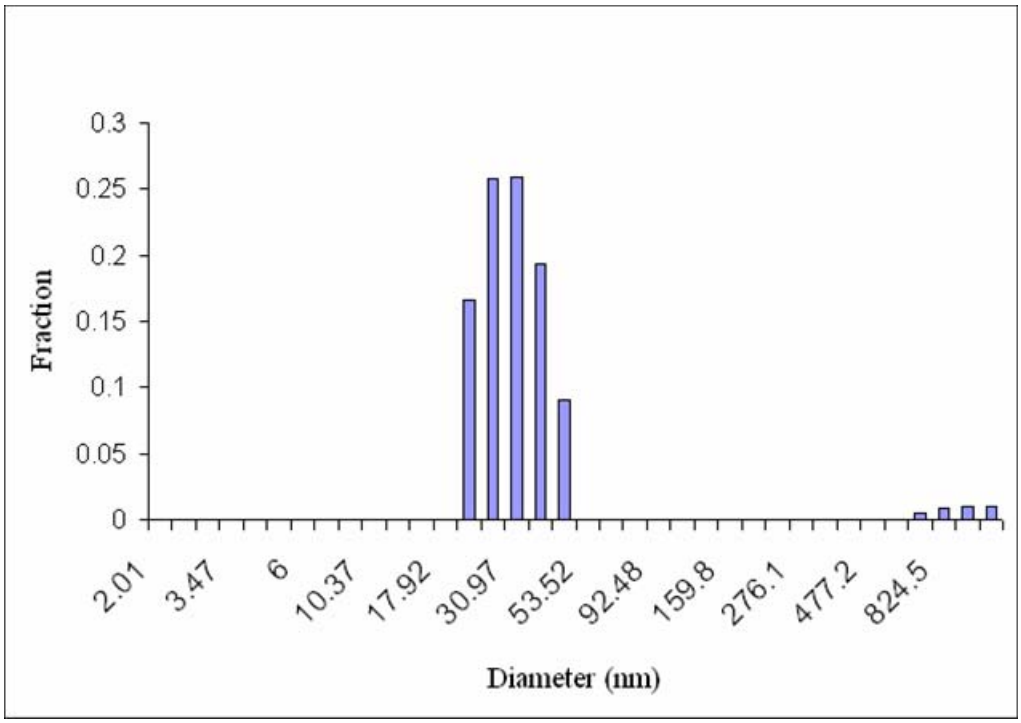

Figure 2.7. Typical DLS scan showing the diameter of DMPC vesicles. Sizes range from 20-60 nm in diameter.

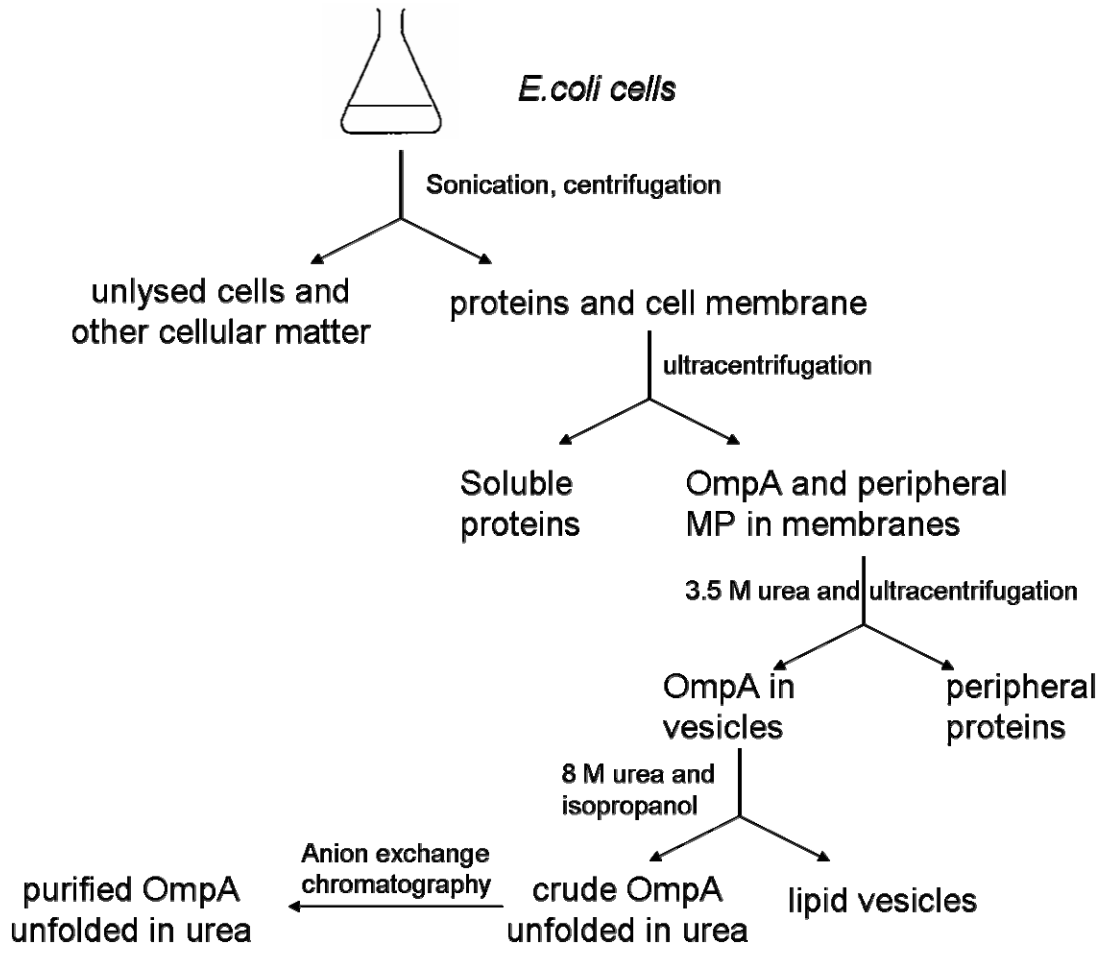

Figure 2.8. Scheme showing the major steps of OmpA extraction from outer membranes.

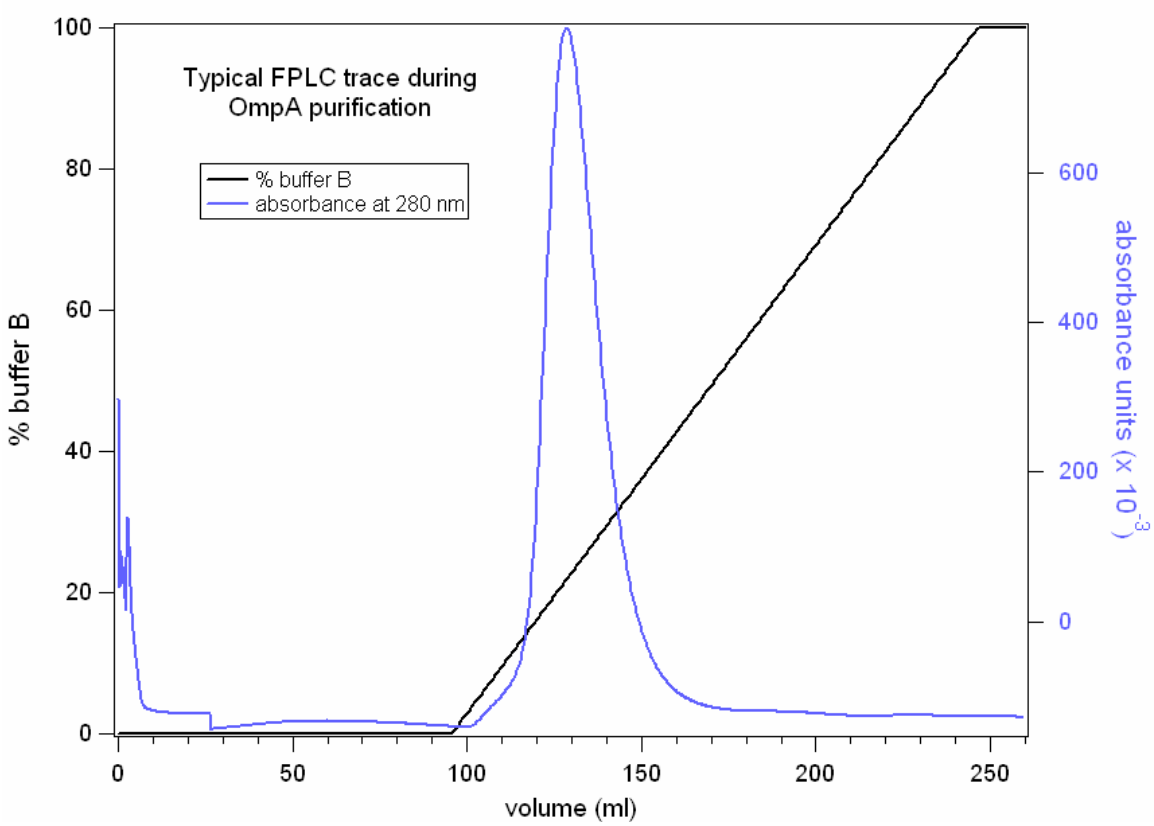

Figure 2.9. Representative FPLC trace for purification of OmpA mutants on a HiTrap QFF column. A 0-200 mM NaCl gradient was used to elute unfolded protein, which comes out as a broad peak on the trace.

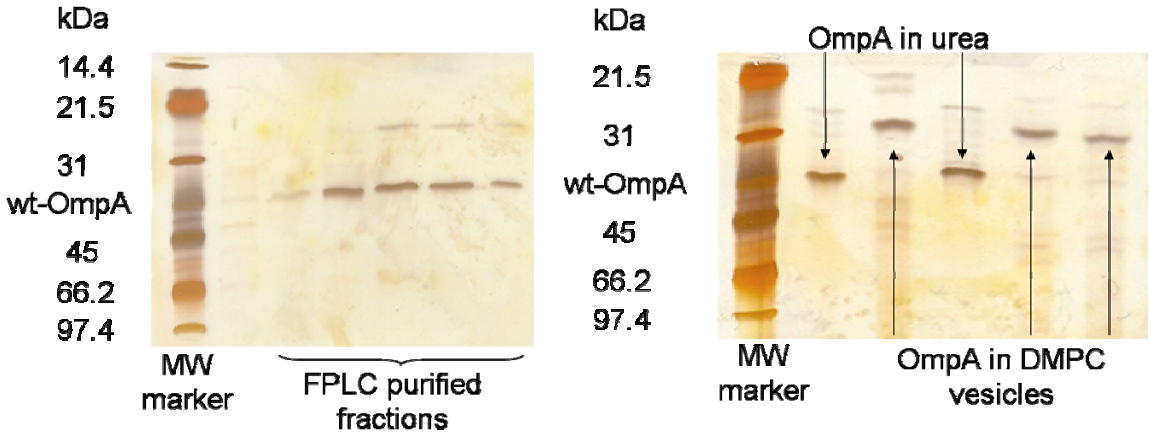

Figure 2.10. Typcial SDS-PAGE of FPLC purified OmpA is shown on the left. The right figure shows unfolded (35 kDa) and folded (30 kDa) OmpA in vesicles.

OmpA protein	Number of residues	Calculated molecular weight (kDa)	Extinction coefficient (M^{-1} cm^{-1})
Wild-type	325	35172	54390
Full-length single Trp mutants (W7, W15, W57, W102, W143)	325	35016	32330
Truncated single Trp mutants (W7t, W15t, W57t, W102t, W143t)	176	19044	26020
W7/C290S/C302S	325	34984	32330
W7/C290S/C302S/A175C	325	35016	32330
W7t/A175C	176	19076	26020
Signal sequence	22	2047	n/a

Table 2.1. List of proteins prepared and their number of residues, calculated molecular weight (kDa), and calculated extinction coefficients.

2.4 CONCLUSIONS

OmpA is a 325 residue monomer that contains 5 native Trp residues in the transmembrane region. Single Trp mutants were produced and the C-terminal domain was removed to produce the 176 residue truncated variants of the single Trp mutants. The expression and purification of 5 full-length and 5 truncated single tryptophan mutants have been successfully accomplished using published protocols with slight modifications. OmpA and its mutants are easily expressed and purified with yields high enough for spectroscopic experiments. DMPC vesicles were also characterized by DLS and confirmed to have diameters of ~20-50 nm, in agreement with published literature.

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