Spectroscopic Studies of Model Protein Interactions with Lipid Vesicles

and

Insulin Fibril Disassembly

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THESIS

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
ATR	Attenuated Total Reflectance
βLG	β -lactoglobulin
CCD	Charge-Coupled Device
DG	n-dodecyl- β -D-glucopyranoside
DLPC	1,2-dilauroyl-sn-glycero-3-phosphocholine
DLS	Dynamic Light Scattering
DM	n-dodecyl- β -D-maltoside
DMPC	1, 2-dimyristoyl-sn-glycero-3-
	phosphocholine
DMSO	Dimethyl Sulfoxide
DOPC	1, 2-dioleoyl-sn-glycero-3-phosphocholine
DSPC	1, 2-distearoyl-sn-glycero-3-phosphocholine
DTGS	Deuterated Triglycine Sulfate
DTT	Dithiothreitol
ECD	Electronic Circular Dichroism
E.coli	Escherichia coli
ESR	Electron Spin Resonance
FTIR	Fourier Transform infrared
ICD	Induced Circular Dichroism
LUV	Large Unilamellar Vesicle

LIST OF ABBREVIATIONS (continued)

MCT	Mercury Cadmium Telluride
MLV	Multilamellar Vesicle
NMR	Nuclear Magnetic Resonance
OG	n-octyl- β -D-glucopyranoside
OMP	Outer Membrane Protein
PAGE	Polyacrylamide Gel Electrophoresis
PEM	Photoelastic Modulator
PLL	Poly-L-Lysine
РМТ	Photomultiplier
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-
	phosphocholine
SDS	Sodium Dodecyl Sulfate
S/N	Signal-to-Noise Ratio
SUV	Small Unilamellar Vesicle
TEM	Transmission Electron Microscopy
TFE	2,2,2-trifluoroethanol
ThT	Thioflavin T
UV-Vis	Ultraviolet-Visible
VCD	Vibrational Circular Dichroism

SUMMARY

The work in this thesis focuses on spectroscopic studies of refolding and interaction of model proteins with various lipid vesicles and on dimethyl sulfoxide (DMSO)-induced disassembly of various structural variants of insulin fibrils.

The main spectroscopic methods utilized in this thesis include electronic circular dichroism (ECD), fluorescence, infrared absorption (IR) and vibrational circular dichroism (VCD). ECD, IR and VCD were used to characterize the secondary structure of model proteins based on their distinct bandshapes for various secondary structure elements. Polarized attenuated total reflectance (ATR)-FTIR was particularly useful in a protein-lipid membrane study (Chapter 3), because it can provide information about relative orientation of protein segments and the lipid bilayer. VCD was used to probe the disassembly of insulin fibrils (Chapter 4) due to its sensitivity to supramolecular chirality arising from higher-order self-assembly of aggregates. Fluorescence was used to monitor protein local tertiary structural change in protein-lipid vesicle interaction.

In Chapter 3, a β -sheet to α -helix transformation of monomeric β -lactoglobulin (β LG) induced by small unilamellar vesicles (SUVs) of zwitterionic lipids at low pH was determined via various spectroscopic techniques. With SUVs of a zwitterionic lipid (1, 2-distearoyl-sn-glycero-3-phosphocholine, DSPC), β LG converted to a substantially helical form in a two-step kinetic process (fast and slow steps) monitored by CD. Fluorescence implied a rapid initial change in the Trp environments followed by a slower process paralleling the secondary structure change. Polarization ATR-FTIR results indicate the helices formed are at least partially inserted into the lipid bilayer and the sheet segments

SUMMARY (continued)

are on the surface. Thermal behavior showed that changes in the secondary structure for the lipid bound β LG occurred in three phases: the first is a slight reduction of the α -helix for β LG in the protein-lipid complex; the second is the DSPC phase change after which the proteins apparently dissociated from the vesicles and refolded into their native structures; the third is the unfolding of solvated β LG at high temperature. These thermal and kinetic behaviors suggest a different mechanism for the monomeric β LG interaction with zwitterionic lipids than was seen previously for the dimeric form at higher pH.

In Chapter 4, VCD was utilized to characterize the macroscopic chirality and the DMSO-induced disassembly process for two types of insulin fibrils formed under different conditions. In this study it is confirmed that very high concentrations of DMSO both disaggregate these insulin fibrils and change their secondary structure. Interconversion of some insulin fibril types also occurred during the destabilization process as monitored by VCD. Transmission electron microscopy (TEM) images correlated the change in VCD sign pattern to alteration of morphology of the insulin fibrils.

In Chapter 5, a computationally designed outer membrane protein (OmpFG) expected to form a monomeric β -barrel in the membrane was expressed and studied. At least partial refolding was evidenced in the lipid vesicles by CD detection of secondary structural change (random coil to β -sheet) and the change to a less polar environment of Trp residues was monitored by fluorescence. The results of a dye leakage assay indicate that the OmpFG can interact with lipid vesicles and may form a pore-like structure with relatively high conductivity.

1. Introduction

1.1 Overview

Proteins are vital building blocks of all biological species. They serve a variety of biological functions, including catalysis, transportation, storage, immune response, and mechanical support.⁽¹⁾ In these biological roles, proteins can interact with other molecules, including other proteins, lipids, nucleic acids, or small molecules. However, unfolding or misfolding of proteins may lead to the loss of protein biological function, protein aggregation, or even cell death, and can cause or be symptomatic of many diseases.^(2, 3) Therefore, it is important to understand the mechanism of protein folding and misfolding. In this thesis, multiple spectroscopic techniques were used to study the interaction of proteins and other molecules, protein folding, aggregation and disaggregation processes by monitoring the associated changes in protein structures, especially secondary structural changes.

This thesis consists of five chapters. The first chapter starts with a brief introduction about protein structures, the mechanism of protein folding and misfolding, protein-lipid interaction and some structural and functional knowledge of studied proteins. Chapter 2 summarizes experimental methods and protocols that used in the thesis studies. Chapter 3 through chapter 5 presents three separate studies forming the core of my research work.

1.2 Protein Structure

Proteins are polypeptides formed by amino acid residues, whose structure is categorized by four levels: primary, secondary, tertiary and quaternary, as shown in Figure 1.1.⁽⁴⁾ Primary structure is the number and sequence of amino acids in the linear

polypeptide chains. Secondary structure refers to the ordered conformations occurring sequentially in polypeptide chains, which are often stabilized by hydrogen bonds between amine hydrogen and carbonyl oxygen atoms of peptide bonds. Tertiary structure is the three-dimensional arrangement of secondary structural elements (helix, sheet and turn or loops). Finally, the overall organization of protein subunits is called quaternary structure. The following parts will be focused on the first three levels of protein structure, which are more related to my thesis work.

1.2.1 Primary Structure

Primary structure is defined as a sequence of mostly L-amino acid residues in a linear arrangement. The carboxylic group of one amino acid is covalently linked with the amino group of another amino acid through a dehydration coupling to form a peptide bond, and all the residues are connected in the same manner; therefore proteins are known as polypeptides. The polypeptide sequence, as synthesized in the cellular system, starts with N-terminal residue and ends with C-terminal residue.

The peptide bond is stabilized by resonance, delocalization of electrons over the O-C-N atoms, which not only increases the polarity of the peptide bond, but also results in its partial double bond character. X-ray crystallography studies on simple peptides have shown that the C-N bond in peptide bond is shorter than a regular single bond.⁽⁵⁻⁹⁾ Moreover, due to the effect of resonance, the O-C-N atoms in the peptide bond are coplanar, resulting in limited free rotation of the bond. As shown in Figure 1.2,⁽¹⁰⁾ there are two main torsion angles, phi (Φ) is the angle around the N-C_a bond and psi (Ψ) is the angle aroud the C-C_a bond. There is another torsion angle omega (Ω), for the rotation about the C-N amide bond, which is restricted by the partial double bond character (not shown in figure). These torsional rotations result in a rotation of entire plane of peptide group, thus they can affect the relative peptide geometry along the polypeptide backbones.

1.2.2 Secondary Structure

Most proteins have compact conformations instead of extended, flexible polypeptide chains. Especially those that contain many hydrophobic amino acid residues prefer to form a tightly packed core to reduce contacts between hydrophobic groups and water molecules. The polypeptide chains can fold into various conformations with certain values of Φ and Ψ torsion angles of the backbone occurring in a repeat pattern. These adopted conformations are defined as secondary structures, including *a*-helix, *β*-sheet and *β*-turns. In 1951, Pauling and Corey first proposed two periodic structures, *a*-helix and *β* pleated sheet.^(7, 11) Other structures like *β*-turns and Ω loops were described later. The allowed values of Φ and Ψ torsion angles can be plotted on a diagram used for classifying different backbone conformations, known as a Ramachandran plot (Figure 1.3).⁽¹²⁾

However, folded proteins may alter their secondary structure, since transitions from one structure to another can occur when conditions are changed, such as pH, temperature, ionic strength and solvent environment or interaction with other molecules. Research focused on such secondary structure transitions will be specifically discussed in Chapter

3.



Figure 1.1 Protein structural organization: primary, secondary, tertiary and quaternary structures. Adapted with permission from "Design of Folded Peptides", Venkatraman, J., Shankaramma, S. C., and Balaram, P., *Chem. Rev.* 2001, 101(10), 3131-3152. Copyright (2001) American Chemical Society.



Figure 1.2 The geometry of peptide bond in polypeptide chain showing the backbone length and angles. The shaded regions are planar peptide groups and the backbone torsion angles are indicated with circular arrows with " Ψ " and " Φ " marked. Adapted with permission from Petsko, G. A., and Ring, D., *Protein Structure and Function*, New Science Press, London, 2004.



Figure 1.3 Ramachandran plot. The combination of backbone torsion angles Φ and Ψ adopted for different residues are shown in dark orange. The red spots are values for common protein secondary structure elements based on average values of Φ and Ψ . The isolated α -helical region on the right is a left-handed helix, and the one on the left is the more common right-handed helix. Adapted with permission from Petsko, G. A., and Ring, D., *Protein Structure and Function*, New Science Press, London, 2004.
1.2.2.1 The α -helical Conformation

The most common secondary structure element in folded proteins is the α -helix, which is stabilized by local hydrogen bonding between C=O and N-H groups of amino acid residues (i, i+4), which are aligned roughly parallel to the helix axis. Pauling and Corey proposed a rodlike peptide structure, called an α -helix (Figure 1.4).⁽¹¹⁾ In the α helix, the hydrogen bonds are formed between every four residues from C-terminal to Nterminal (NHⁱ⁺⁴ to COⁱ). According to the specific values of residues per turn (n), the helix pitch (h) and torsion angle values (ψ , ϕ , and ω), helix can be further classified into several types (Table 1.1). Normal α -helix has 3.6 residues per turn with a pitch of 1.5 Å, while 3₁₀-helix (i, i+3) has a narrower helical structure and π -helix (i, i+5) is wider and shorter based on the helical parameters. Moreover, an α -helix can be amphipathic, with hydrophilic and hydrophobic groups distributed 3-4 residues apart, in which case, it will tend to have one side with hydrophilic residues and the other with hydrophobic ones.⁽¹⁾

Two shapes can be obtained for an α -helix, right-handed with clockwise direction or left-handed with counterclockwise direction, however the right-handed one is more favored because there are less steric constraints between the side chains and backbone for L-amino acids by forming this shape. Thus almost every α -helical structure found in proteins is right-handed.

Due to properties of amino acid side chains, only some amino acids have high preference to form helical structure, such as Glu, Ala, Leu, Met, Gln, and Lys.⁽¹³⁾ Many proteins existing in muscle, blood clots and hair have very stable coiled coils of α -helical structure formed by more than two α -helices.⁽¹⁾ Some proteins in biological membranes also contain α -helical structures or bundles of helices.



Figure 1.4 Structure of α -helix. (A) The ribbon diagram indicates the right-handed α -helix with α -carbons (grey dot) and side chains (green) shown in color. (B) The side view shows the hydrogen bonds (green dash line) between NH and CO groups. Adapted with permission from Berg, J. M., Tymoczko, J. L., and Stryer, L., *Biochemistry* 5th edition, W. H. Freeman, New York, 2002.

Conformation	Torsion Angles			Residues/turn	Translation/residue
	ϕ	ψ	ω		(Å)
α-helix	-57	-47	180	3.6	1.5
3 ₁₀ helix	-49	-26	180	3.0	2.0
π-helix	57	-70	180	4.4	1.15
Polyproline I	-83	+158	0	3.33	1.9
Polyproline II	-78	+149	180	3.0	3.12
Polyproline III	-80	+150	180	3.0	3.1

 Table 1.1 Table of helical parameters.

Adapted with permission from Petsko, G. A., and Ring, D., *Protein Structure and Function*, New Science Press, London, 2004.

1.2.2.2 The β -sheet Conformation

The other protein secondary structure proposed by Pauling and Corey is the β -sheet, which is stabilized by hydrogen bonds between an extended polypeptide chain and a neighboring chain.^(7, 14) In the β -sheet, each interacting extended polypeptide chain is called a β -strand, and two or more β -strands are arranged side by side with hydrogen bonds, to form a β -sheet. If the adjacent β -strands run in the same direction, they can form a parallel β -sheet, or in the opposite direction they can form an antiparallel β -sheet (Figure 1.5). The hydrogen bonds in an antiparallel β -sheet are more linear than in a parallel β -sheet. The distance between neighboring residues in parallel and antiparallel β sheets is 3.2 and 3.4 Å, respectively.

In a β -sheet, almost all the N-H and C=O groups are hydrogen bonded, except the outside ones on strands at the edge of sheets. There are several ways for the edge strand to form hydrogen bonds. 1) If they are solvent exposed, hydrogen bonds can be formed with water molecules. 2) They can link to the edge of another protein chain by hydrogen bond to form an extended β -sheet structure. 3) The sheet may curve itself, with two edge strands linked to each other forming a closed cylinder structure, so called β -barrels, which is a very common protein structure, especially for membrane proteins. A class of outer membrane proteins with essential biological functions, called porins, is dominated by β -barrel structure, and will be specifically addressed in Chapter 5.



Figure 1.5 The structures of β -sheet. (a) Mixed parallel and antiparallel β -sheet structures. The hydrogen bonds are shown in red dash lines. (b) Edge views of antiparallel (top) and parallel β -sheets (bottom). Adapted with permission from Petsko, G. A., and Ring, D., *Protein Structure and Function*, New Science Press, London, 2004.

1.2.2.3 The β -turn and Loop Conformations

The reversal of the polypeptide chain direction will result in the formation of loops or reverse turns that occur mostly in globular shaped proteins. As shown in Figure 1.6, in a β -turn, the C=O group of residue *i* forms a hydrogen bond with the N-H group of residue *i*+3 in the reverse direction.⁽¹⁵⁾ The structures of loops (also called Ω loops), are longer; however, they are not uniform and do not adopt periodic structures like α -helix or β -sheet. According to the numbers of amino acids involved, the turns can be classified into several types: γ -turns (three residues), β -turns (four residues), α -turns (five residues) and π -turns (six residues). Some amino acid residues tend to occur in reverse turns with high probability, due to their specific restricted values of torsional angles, like Pro, or due to the wide range of torsion angles, like Gly.⁽¹³⁾ Therefore, the sequence of Pro-Gly or Gly-Pro sometimes can be used to recognize turn structures.⁽¹⁶⁾

Turns and loops often exist on the surfaces of proteins, thus they can be involved in various protein-protein or protein-other molecule interactions and can play important roles in protein folding.⁽¹⁷⁾ Moreover, when the turns are exposed to the solvent, they can form hydrogen bonds with water molecules and help to maintain the compact protein structure in the aqueous environment.

1.2.2.4 The Disordered Structure (Random Coil-like)

Proteins in non-native states have been widely studied due to their relevance to protein folding, stability and function. When the backbones of a protein or peptide coil randomly without adopting any regular secondary structure, it forms a disordered conformation.⁽¹⁸⁾ Based on analyses of statistic distributions of Φ and Ψ , disordered structures contain disordered residual structures in denatured proteins and peptides.⁽¹⁹⁾



Figure 1.6 Structures of β -turn (top) and loops (bottom, shown in red). Adapted with permission from Berg, J. M., Tymoczko, J. L., and Stryer, L., *Biochemistry* 5th edition, W. H. Freeman, New York, 2002.

Proteins can adopt disordered conformations under denaturing conditions, such as above melting temperature or in strong denaturants (e.g. guanidinium chloride or urea).^(20, 21)

Many studies showed that disordered peptides and denatured proteins contain some local structures with a high probability for a characteristic left-handed turn, called the polyproline II helix (PPII).^(22, 23)

The disordered conformation can be detected by various spectroscopic techniques, such as CD and FTIR via distinct band shapes and peak positions. Thesis work about spectroscopically monitored protein denaturation will be addressed in the Chapter 4.

1.2.3 Tertiary Structure

For compact folded proteins, several regular secondary structure elements can interact with each other with their hydrophobic and hydrophilic groups interacting to form a more compact structure, which is called tertiary structure. Tertiary structure is the overall shape of a polypeptide, which is stabilized by various intramolecular or intermolecular interactions between the side groups of polypeptide chains, including electrostatic interaction between ionic groups, hydrophobic interaction, hydrogen bond formation and van der Waals interactions. Moreover, the sulfhydryl groups on the Cys amino acid side chains can form disulfide bonds to bring separated parts of the polypeptide chains together.

The secondary structure elements can be packed in different ways, showing various topologies. Based on those topologies, tertiary structure can be classified into several types.

1) All- α topologies: some small proteins or peptides contain more than one α -helix segment bound together or they have two helices connected by a short loop (helix-turn-

14

helix).

2) All- β topologies: some folded proteins contain almost all β -sheets in a mostly antiparallel arrangement.

3) α/β topologies: a common structure exists in many enzymes, showing a repeat $\beta\alpha\beta$ units.

4) $\alpha + \beta$ topologies: domains contain separate α -helix and β -sheet regions, particularly antiparallel β -sheet regions.

5) Small disulfide-rich folds: some small proteins contain a large number of disulfide bonds to stabilize the folded structures.

1.3 Protein Folding and Misfolding

The self-assembly of component structures in living systems is very precise and well-defined. One of these self-assembly processes is protein folding. Understanding its mechanism is very important but also challenging to modern science.^(3, 24) Protein folding is the process by which extended polypeptides in the unordered state fold into their compact three-dimensional structures. However, it would take too long for unordered polypeptides have an unbiased search over all possible conformations during the folding process.⁽²⁵⁾ Thus the protein folding process should follow specific pathways which can simplify the folding mechanism into several steps.

There are three common models to describe the protein folding process, as shown in Figure 1.7.⁽²⁶⁾

1) Framework model, in which the secondary structures form first, and then the preformed secondary structure elements connect to each other through long-range interactions to form a stable tertiary structure.⁽²⁷⁻³⁰⁾

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2) Hydrophobic collapse model, in which the protein adapts a compact conformation with hydrophobic residues buried inside and hydrophilic residues on the surface. In this way, protein folding can occur within a confined conformation.⁽³¹⁻³³⁾

3) Nucleation condensation model, is the combination of the other two models, in which the protein collapses to reduce the distance between amino acids, leading to the formation of secondary and tertiary structure at the same time.⁽³⁴⁾ Thus folding can take place in one step without forming intermediates. Moreover, this combined model can shift to either of the other models by changing the stability of relevant secondary or tertiary structures.⁽²⁶⁾

If a protein fails to form a correct native structure (misfolding), it will lose biological function or go through severe impairments, such as translocation across membranes, secretion, trafficking or immune response.^(3, 35) There are several pathways in the protein folding and misfolding process, and most of them are reversible, except the ones that form very stable aggregates (Figure 1.8).⁽³⁾ These aggregated structures, especially amyloid fibrils, are related to several diseases. In the fibril state, misfolded proteins typically form similar cross- β structures with fibrillar morphology regardless of the amino acid sequences.⁽³⁶⁾



Figure 1.7 Schematic view of three protein folding models: framework, hydrophobic collapse and nucleation condensation. Adopted with permission from "Is There a Unifying Mechanism for Protein Folding?", Daggett, V., and Fersht, A. R., *Trends Biochem. Sci.* 2003, 28, 18-25. Copyright (2002) Elsevier.



Figure 1.8 Structures formed in folding and misfolding processes. Adapted with permission from "Protein Folding and Misfolding", Dobson, C. M., *Nature* 2003, 426, 884-890. Copyright 2003 Nature Publishing Group.

1.4 Protein - Lipid Interaction

Although proteins are synthesized in the same manner, not all the folded proteins in the cell are stable in an aqueous environment. Some are fully or partially buried in the hydrophobic interior of a membrane. Biological membranes, in particular on the surface of the cell, are formed by bilayers of lipid molecules, which have hydrophobic tails (hydrocarbon chains) and polar head groups. Proteins that embed in the membrane lipid bilayer are called membrane proteins and usually contain many hydrophobic residues forming transmembrane segments. Membrane proteins are commonly classified into two types, α -helical bundle proteins and β -barrel proteins based on their main secondary structures.⁽³⁷⁻³⁹⁾

The hydrophobic interior of a lipid bilayer is about 30 Å thick, and the head groups occupy around 5-10 Å on each side of the membrane.⁽¹⁰⁾ The structures of a few lipid molecules have been resolved using high-resolution X-ray, for example, the bilayer form factors of dioleoylphosphatidylcholine (DOPC) [di(C18:1)PC] in the fluid phase (L_{α}) were determined by X-ray and neutron diffraction techniques.⁽⁴⁰⁾ As shown in Figure 1.9, the most rigid part is the glycerol backbone and has the narrowest motional region dispersion, and the motion increases for head groups and fatty acyl chains, which are highly involved in the protein-lipid interaction.^(37, 41)

In terms of folding driving force and kinetic pathways, the folding and insertion mechanisms for α -helical bundle and β -barrel proteins are quite different (Figure 1.10).⁽⁴²⁾ Due to the difficulties of *in vivo* study, the folding mechanism of purified membrane proteins can be studied *in vitro* instead, using various techniques, such as EPR, FTIR, fluorescence and MD simulations.



Figure 1.9 The structure of a bilayer made by DOPC at 23 °C and low hydration. The structural fragments are described by Gaussian distribution. The position and width of the Gaussians represent the probable location and thermal motion range of DOPC fragments, respectively. Adapted with permission from "Lipid-Protein Interactions in Biological Membranes: A Structural Perspective", Lee, A. G., *Biochim. Biophys. Acta.* 2003, 1612, 1-40. Copyright 2003 Elsevier.



Figure 1.10 The folding and insertion mechanism of helical bundle (left) and β -barrel proteins (right) based on *in vitro* folding studies. Adapted with permission from Luckey, M., *Membrane Structural Biology with Biochemical and Biophysical Foundations*, Cambridge University Press, New York, 2008.

There is a widely accepted two-stage model for the folding and insertion of α -helical bundle proteins.⁽³⁸⁾ Stage I is the helix insertion step, which is driven by the hydrophobic effect between hydrophobic side chains of protein amino acid residues and the lipid bilayer interior. Based on thermodynamic analysis, White and Wimley proposed that the stage I can be further separated into three sub-stages: partitioning, folding and insertion.⁽⁴³⁾ The hydrophobic side chains of the protein provide enough free energy for lipid perturbation and helical partitioning, which can induce α -helical folding. Stage II is the assembly of helices, which is driven by other factors, including packing, electrostatic effects and interaction between loop regions of protein and lipid head groups on the membrane surface. For some proteins, the folding process needs a third stage, which includes rearranging the loop regions or prosthetic groups.⁽⁴⁴⁾

Unlike the α -helical bundle proteins, whose individual helices can be formed independently, β -barrel proteins need at least three to five β -strands forming hydrogen bonds between each other to stabilize the whole structure. Thus β -barrels require all the strands to be formed at about the same time.⁽⁴²⁾ An *in vitro* folding study of β -barrel proteins showed that the folding process starts in the aqueous environment and is coupled with the step of the insertion of folded protein. ⁽⁴⁵⁾ A folding study on some model β barrel proteins will be specifically addressed in Chapter 5.

Protein-membrane interactions not only happen between lipids with membrane proteins, but also with some other soluble proteins. The driving forces of this proteinlipid interaction are mostly electrostatic interaction, hydrophobic effect or both.

1.5 Model Proteins

It is difficult to directly study the folding and misfolding process of protein in biological environments, however, *in vitro* studies on some model proteins can be used to provide new insight into these processes.

1.5.1 Bovine β -Lactoglobulin

 β -lactoglobulin is a major whey protein from lipocalin protein family,⁽⁴⁶⁾ and it has been reported to be present in the milk of many but not all, mammalian species, such as cow, horse and cat, but rodent, human and lagomorph milks are exceptions that have no β LG.⁽⁴⁷⁻⁴⁹⁾ The lipocalins usually have multiple functions, most of which are related to ligand-binding, however, the function of β LG is still unclear.⁽⁵⁰⁾ Since β LG exists in cow's milk in significant quantities, it has been studied under physiological conditions to understand its properties and biological function. It was reported to be related to aggregation and denaturation during milk processing.⁽⁵¹⁾

 β LG is a small soluble protein having 162 amino acid residues with a molecular weight ~ 18400 g/mol. In the native state, it adopts a flattened β -barrel structure containing 8 antiparallel β -sheet strands and a 3-turn α -helix on the outer surface to form a hydrophobic core, which can provide a ligand-binding site for hydrophobic molecules.⁽⁵²⁾ It exists as a dimer at neutral pH but dissociates into monomers at pH 3 and below.^(53, 54) As shown in Figure 1.11, the calyx, or β -barrel is made by one antiparallel β sheet formed by strands A-D facing to the other sheet formed by strands E-H.⁽⁵²⁾



Figure 1.11 Ribbon diagram of a single subunit of β -lactoglobulin. Adapted with permission from "Bovine β -lactoglobulin at 1.8 Å Resolution Still an Enigmatic Lipocalin", Brownlow, S., Cabral, J. H. M., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., North, A. CT. and Sawyer, L., *Structure* 1997, 5, 481-495. Copyright (1997) Elsevier.

The loops close to the end of the barrel are quite short, like loop BC, DE, and FG, and those at open end are longer and flexible which can be involved in many interactions. The loop AB, along with strand I, can form a dimer interface, which can have hydrogenbonding interaction with the same loop of a second monomer.⁽⁵²⁾ Actually both the loop AB and strand I are essential to the dimerization of β LG, modification or mutation on either one will result in no dimer being formed at physiological pH.⁽⁵⁵⁾ The EF loop located at the open end of the calyx, can have a conformational change depending on pH, known as a Tanford transition.⁽⁵⁶⁾ The crystal structure and NMR study showed that the EF loop is oriented in a position to "close" the hydrophobic binding site of the calyx at pH 6.2 and in an "open" position at pH 7.1.^(57, 58) β LG contains two disulfide bonds formed by Cys 106-Cys 66 and Cys 106-Cys 119 and one free thiol group, Cys 121, which can be involved in denaturation and aggregation behavior because of its pHdependent activity.⁽⁵¹⁾ Additionally, two tryptophan residues, that exist in the monomeric β LG can be detected by fluorescence; Trp 61 is exposed to the solvent, while Trp 19 is buried in the hydrophobic core.⁽⁵⁹⁾

It was reported that β LG can bind many ligands specifically, such as surfactants,⁽⁶⁰⁾ palmitic acid,⁽⁶¹⁾ cholesterol⁽⁶²⁾ and other hydrophobic molecules.^(63, 64) A variety of techniques were used to investigate these protein-ligand interactions, including electron spin resonance spectroscopy (ESR), spectrophotometry, perturbation of intrinsic tryptophan fluorescence and NMR.^(47, 61, 65) The hydrophobic pocket inside the β -barrel of β LG can provide a main ligand-binding site ⁽⁵⁰⁾ Additionally, many studies showed that there may be more than one binding site on the β LG monomer; however, some sites are too weak to be located.⁽⁶⁶⁻⁷¹⁾

The secondary structure of native β LG is dominated by β -sheet, but it has a great propensity to adopt α -helix structure, since it undergoes α - β transition, changing from α helical intermediate to β -sheet in the native state, during its folding process. ⁽⁷²⁻⁷⁶⁾ The native β LG can have β - α transition and obtain nonnative α -helical structure in the presence of 2,2,2-trifluoroethanol (TFE)⁽⁷²⁾, charged surfactant⁽⁷⁷⁾, alcohol⁽⁷⁸⁾ and phospholipid bilayers,⁽⁷⁹⁾ under physiological conditions. The Keiderling group previously reported that β LG can have a β - α transition by interacting with anionic lipid vesicles at physiological and acidic pH (pH 6.8 and pH 4.6, respectively).⁽⁸⁰⁻⁸²⁾ Their equilibrium and kinetic studies revealed that the main driving force of this transition is the electrostatic interaction between the positively charged protein and negatively charged lipid, and that the hydrophobic effect plays a secondary role to develop the α helical conformation.

At low pH, β LG is dissociated into a monomer form that maintain a stable β -sheet secondary structure. It was reported that, at low pH, β LG can also have β - α transition induced by highly concentrated methanol forming significant α -helical secondary structure with a molten globule tertiary structure characteristic.⁽⁸³⁾ The molten globule state is a "thermodynamic state", that is different from both the native and unfolded states. Molten globule forms of proteins often are in partially unfolded states under mildly denaturing conditions, that maintain some secondary structures (native-like or other), but have a dynamic tertiary structure. These changes can be monitored by far-UV CD for secondary structure and collapse of near-UV CD (of aromatics) for tertiary structure.⁽⁸⁴⁾ The secondary structural transition of β LG at low pH induced by interaction with lipid vesicles will be discussed in Chapter 3.

1.5.2 Bovine Insulin

Insulin is a peptide hormone synthesized in the beta cells of the pancreas, and is comprised of two polypeptide chains A and B connected by disulfide bonds, with the chains having 21 and 30 amino acid residues, respectively (Figure 1.12a).⁽⁸⁵⁾ The amino acid sequence of insulin is very conserved, for example, bovine insulin only has three residues different from human insulin. Insulin is stored in the body as a hexamer, although its active form is a monomer with a significant α -helix secondary structure (Figure 1.12b).

The main function of insulin is to regulate the metabolism of carbohydrates and fats by promoting the absorption of glucose from blood. The failure of its function or lack of its production will result in diabetes mellitus disease.⁽⁸⁶⁾

Because soluble bovine insulin can convert to fibril forms under appropriate conditions such as low pH, high temperature and high ionic strength,⁽⁸⁵⁾ it provides a good model protein to study the formation and disaggregation of amyloid aggregates. Like other amyloid fibrils, insulin fibrils have a β -sheet secondary element as a core structure that elongates along the fibril axis, which is an interpretation supported by early X-ray diffraction, circular dichroism (CD) and infrared spectroscopy (FTIR) studies.^(87, 88)

In Chapter 4, a disaggregation study on various model insulin fibril types will be discussed in detail.



Figure 1.12 The primary structure and quaternary arrangement of insulin. (a) The amino acid residues and disulfide bonds are indicated for chain A and B of insulin. (b) Chain A (orange) and chain B (blue) of insulin are assembled by post-translational modification including disulfide bridge formation (yellow). The side view and top view of assembled structures are shown on the right.

Adapted from "The Structure of Insulin", Cartailler, J. P., Beta Cell Biology Consortium (BCBC), <u>https://www.betacell.org/content/articleview/article_id/8/</u>.

1.5.3 Outer Membrane Protein

Gram-negative bacteria are protected by two lipid bilayer membranes, the inner and outer membranes with a periplasmic space in between. Both membranes contain many proteins, however, their composition and function are different. The inner membrane is symmetric consisting of the same phospholipids on each side. mainly phosphatidylethanolamine (PE-lipid), while the outer membrane is quite asymmetric, containing additional lipopolysaccharides on the outer leaflet.⁽³⁹⁾ Among the proteins existing in the outer membrane, one class of pore-forming proteins (porins) has been studied a lot recently due to its specific function of forming diffusion channels to allow the rapid diffusion of solute through the membrane.⁽⁸⁹⁾

Based on the selectivity of the diffusion channels, porins can be classified into two groups: 1) General porins, such as OmpF and OmpC, can form channels that have no specific selectivity for various small, hydrophilic solutes (< 600 Da). 2) Specific porins, including PhoE, LamB, ScrY and Tsx, form channels with specific selectivity.⁽⁹⁰⁾

OmpF was the first membrane protein to have its structure determined by X-ray crystallography.⁽⁹¹⁾ It forms homotrimers of around 115 kDa in the outer membrane, and each monomer consists of 16 β -strands. Most of the long and extended loops of OmpF are exposed at the cell surface, however, the third loop, L3, folds into the barrel interior causing a constriction region around the center of the channel (Figure 1.13).^(39, 90)



Figure 1.13 Structure of OmpF porin. (a) Ribbon diagram of OmpF monomer with most long loops facing exterior and L3 loop (dark) folding inside of the barrel. (b) The top view of the OmpF trimer showing the inside of each pore is constricted by the L3 loop. (c) The pore dimensions are shown by slicing through the center of the OmpF trimer. Adapted with permission from "General and Specific Porins from Bacterial Outer Membranes", Schirmer, T., *J. Struct. Biol.* 1998, 121, 101-109. Copyright 1998 Academic Press.

Another porin protein, OmpG from *E. coli* K-12 has also been widely studied due to its special properties. Based on single-channel conductance results, it seems that OmpG is stabilized and functional as a monomer with 14 β -strands (Figure 1.14).^(92, 93) Although OmpG monomer has fewer strands than some other monomer porins, it can form a channel with an unusually large diameter according to liposome-swelling assay results.⁽⁹⁴⁾ The formation of this larger channel may be related to the lack of long L3 loop which causes a restriction region in other porins.⁽⁹⁵⁾ Moreover, some studies showed that OmpG experiences pH-dependent or voltage-dependent gating processes.^(92, 96)

Porins have distinct characteristics in terms of pore size and conductivity. It would be useful to have an engineered porin designed to combine multiple characteristics of different porins, for example a specifically engineered porin pore could be used as a sensor for single-molecule detection.⁽⁹⁷⁾ A newly designed porin, which combines the OmpF and OmpG sequence, and forms a new diffusion channel, will be discussed in Chapter 5.



Figure 1.14 Overview of OmpG structure. The ribbon diagram represents OmpG monomer structure from the side in the plane of membrane (top) and from extracellular side (bottom) with strands in green, extracellular loops in orange, periplasmic turns in cyan and aromatic residues in grey. Adapted with permission from "Crystal Structure of the Monomeric Porin OmpG", Subbarao, G. V., and van den Berg, B., *J. Mol. Biol.* 2006, 360, 750-759. Copyright 2006 Elsevier.

2. Material and Methods

2.1 Overview

There are various biophysical techniques that can be used for protein structure determination and analysis. However only few can provide detailed three-dimensional structural information about the protein, such as X-ray crystallography, NMR spectroscopy and electron microscopy (especially Cryo-EM). Every method has its own advantages and disadvantages, thus multiple techniques are needed for collecting information to create the final atomic model. Among these methods, X-ray crystallography with special resolution around 0.1 nm or 1 Å has made the greatest contributions to protein structural determination. It can provide atomic resolution to resolve detailed protein structures with high accuracy. The first X-ray photograph from a pepsin crystal was reported by Bernal and Crowfoot in 1934. (98) Until now, most of protein structures included in the Protein Data Bank were determined by X-ray crystallography.⁽⁹⁹⁾ However, X-ray crystallography also has limitations in structure determination: (1) X-ray crystallography cannot locate hydrogen atoms most of the time due to their low electron densities, except by modeling; (2) The locations of some important atoms in the binding site or surface residues with flexible side chains are hard to identify; (3) Crystallization conditions for some proteins may be very different from their physiological conditions, which may affect the prediction of real protein-ligand complexes; (4) There is difficulty in identifying and locating the water molecules.⁽¹⁰⁰⁾ Moreover, X-ray crystallography requires the protein to be crystallized, which may be very difficult for large and insoluble species like membrane proteins.⁽¹⁰¹⁾ The process of

optimizing the crystallization condition and solving protein structure is also timeconsuming and effort-intensive.

Being different from X-ray crystallography, NMR is able to determine the threedimensional structures of protein molecules in both solution and solid phase. Additionally, it is very valuable for studying the dynamics of proteins and other macromolecules which are hard to crystallize.⁽¹⁰²⁾ However, for large proteins, NMR requires isotope labelling to resolve overlapping peaks in the spectra, and their faster relaxation of nuclear magnetization causes broader and weaker NMR peaks. Thus, NMR spectroscopy has been limited in terms of protein size, so that relatively small proteins and protein domains are preferred.

For protein secondary and tertiary structure study, various optical spectroscopic approaches can be used. Most of the methods require only small amount of protein and peptide samples. Optical spectroscopy can also be used to monitor the dynamics of protein or peptide structural changes in the rapid folding/unfolding process or interactions with other molecules. Optical spectroscopy also has limitations that it can only determine average structures of samples with low spatial resolution, but cannot provide site-specific structural information. Since each optical spectroscopic method has its distinct advantage and disadvantage, it is important to combine them to get complementary structural information.

Many biophysical techniques for protein structural characterization related to my thesis work are introduced in this chapter. A general description and some detail about the theory, instrumentation and protocols for dynamic light scattering (DLS), electron circular dichroism (ECD), vibrational circular dichroism (VCD), FTIR, UV-vis and fluorescence will be presented.

2.2 Lipid Vesicles

Due to the difficulties in the study of membrane protein within an integral membrane, reconstitution of cell membranes in an *in vitro* model system has been widely used for biological process studies involving membrane proteins, including transportation, metabolic pathways and single transduction.⁽¹⁰³⁾ A wide variety of model membrane systems have been reported for different applications, for example, monolayers, planar bilayers, supported bilayers, lipid vesicles, micelles, bicelles and nanodiscs. In my thesis, I will only focus on small and large unilamellar vesicles (SUV and LUV) methods.

2.2.1 Mechanism of Vesicle Formation

Lipids can form lamellar bilayer structures over most parts of their phase diagram⁽¹⁰⁴⁾, and can also self-assemble into vesicles in dilute solutions. The formation of vesicles is a two-step process, 1) the amphiphilic lipid forms a bilayer stabilized by hydrophobic effect and the favorability of minimizing the hydrophobic-hydrophilic interfacial area, 2) the bilayer closes to form a vesicle shape due to the tendency for the hydrophobic acyl chains to stay away from water.⁽¹⁰⁵⁾ After dispersion into an aqueous medium, the bilayer edges start to seal and encapsulate the aqueous compartment inside of vesicle during the closing process. Depending on the method used, lipids can form unilamellar (single bilayer) or multilamellar vesicles with variable sizes. The structures and dimensions of three types of vesicles are shown in Figure 2.1.

Multilamellar vesicle (MLV)



Figure 2.1 The structures and dimensions of multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs).

The multilamellar vesicles (MLVs) have many bilayers and encapsulate aqueous medium between the bilayers. The small unilamellar vesicles (SUVs) have smaller size but larger curvature than large unilamellar vesicles (LUVs), thus SUVs have more loosely packed acyl chains.

2.2.2 Lipid Vesicles Preparation Protocol

Lipid vesicles were prepared following a protocol described in literature.⁽¹⁰⁶⁾ The weighed amount of dried lipid (20-30 mg) is transferred into a 25 ml round bottom flask. A proper amount of organic solvent (10-20 mg lipid/ml organic solvent) is then added to solubilize the lipids, chloroform is used for zwitterionic lipids and a chloroform/methanol solvent mixture (2:1, v/v) for charged lipids. When the lipids are thoroughly dissolved and mixed in the organic solvent, the solvent can be carefully removed by evaporation using a mild stream of nitrogen gas while being gently shaken and rotated to yield a thin lipid film. The lipid is further dried under vacuum for several hours or overnight. The lipid film is hydrated by addition of the desired corresponding buffer or solvent which should be pre-warmed at a temperature above the lipid gel-liquid crystal transition temperature (T_m), to yield a final concentration of 12 mM. After addition of hydrating solution, the lipid suspension is well mixed by shaking at 100 rpm to form heterogeneous large MLVs while maintaining the temperature above the Tm during the whole process. Small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) can be obtained by disrupting MLV suspensions using different techniques.

SUVs with a distribution of radii around 20-30 nm determined by dynamic light scattering (which will be described in this chapter) can be made by sonicating MLV suspensions with several 30 sec pulses using a probe tip sonicator until a transparent

solution is formed. Since the probe tip sonication provides a high energy input, the lipid suspensions need to be immersed in a water bath to prevent overheating and maintain the temperature above the lipid phase transition (T_m) .

LUVs can be formed by extrusion, in which the lipid suspension is forced to pass through a defined pore size polycarbonate filter to yield vesicles which will have sizes similar to the pore size. The MLV suspensions are disrupted into smaller size particles by three freeze-thaw cycles prior to extrusion. The disrupted MLV suspensions are then forced to slowly pass through the filter membrane at least 21 times to acquire a transparent solution with uniform vesicle size. During the extrusion step, the temperature should be maintained above that of the lipid phase transition (T_m) to prevent the filter membrane from fouling with rigid lipid particles. The particle sizes of freshly prepared SUV and LUV solutions can be determined with dynamic light scattering (DLS).

2.3 Dynamic Light Scattering

2.3.1 Overview

Dynamic light scattering (also referred as photon correlation spectroscopy or quasielastic light scattering) is a physical technique that can be used to measure the size of suspended small particles or polymers in the solution.⁽¹⁰⁷⁾ With appropriate modification or accessories, DLS can also be used to measure the zeta potential of colloidal dispersions⁽¹⁰⁸⁾, and estimate the molecular weight of organic compounds. In this thesis, DLS was utilized to determine the size distribution of lipid vesicles (SUV and LUV).

2.3.2 Principle and Instrumentation of DLS

When monochromatic light from a laser source hits the small particles, the intensity of scattered light fluctuates over time caused by Brownian motion of molecules and particles in solution. The distance between small particles in the solution changes periodically, thus the scattered light from each particle can interfere constructively or destructively over time (Figure 2.2a), resulting in enhanced or decreased intensity, respectively. Moreover, as shown in Figure 2.2b, smaller particles give rise to more rapid fluctuation of the scattered light intensity than do larger particles.

After processing of light intensity fluctuation by the instrument correlator, the particle size is given in terms of hydrodynamic radius (R_H), which is based on Stokes-Einstein equation, as following:

$$R(H) = \frac{kT}{6\pi\eta D} \tag{2.1}$$

k: Boltzmann's constant

- *T*: absolute temperature
- η : dynamic viscosity

D: translational diffusion coefficient, which is proportional to velocity of Brownian motion and correlated with the fluctuation of scattered light.

A DynaPro Titan dynamic light scattering instrument (Wyatt Technology) with temperature controller was used in this thesis. It contains a laser source, temperature controlled sample compartment and correlator. The sample is illuminated by a semiconductor laser of wavelength at ~ 830 nm, and then the scattered light collected at 90° is detected and processed in the instrument correlator. After autocorrelation of light

intensity fluctuation, the results are sent to the PC for analysis using the DYNAMICS software.

2.3.3 DLS Experiment Protocol

Prior to the measurement, the lipid vesicles need to be filtered or centrifuged to remove dust particles and the sample compartment should be pre-warmed at a temperature above the lipid T_m . The cuvette with 100 µl of sample was then placed into the sample compartment for the measurement. After 30 seconds of laser warm up time, the data were collected every 10 seconds up to 100 acquisitions. Reasonable measured hydrodynamic radius (R_H) values were selected based on those having SOS values (sum of squares difference between the measured data and the cumulants-calculated intensity correlation curves) of less than 20, which is the sum of squares difference between the measured and calculated intensity correlation curves, and these values were averaged.



Figure 2.2 (a) Phase addition of scattered light. (b) Dynamic light scattering of large particles (top) and small particles (bottom).

2.4 Electronic Circular Dichroism

2.4.1 Principles and Applications of ECD

When a light beam is separated into two linearly polarized components (vertical and horizontal) and shifted out of phase by a quarter-wave, the electric field vector traces out a helix in space known as circularly polarized light. It can be either left-handed or right-handed caused by retarding either the horizontally or vertically polarized component. When light passes through an optically active substance, the right-handed and left-handed circular polarized light can travel at different speeds leading to optical rotation. Additionally, the chiral molecules containing one or more chromophores absorb the two polarizations to a different extent. The difference in absorption (ΔA) of left-handed (A_L) and right-handed (A_R) circularly polarized light is called circular dichroism.⁽¹⁰⁹⁾ The measured (ΔA) and molar CD ($\Delta \varepsilon$) can be described using the following equations:

$$\Delta A = A_{\rm L} - A_{\rm R} \tag{2.2}$$

$$\Delta A = (\epsilon_L - \epsilon_R)cl \tag{2.3}$$

$$\Delta \epsilon = \epsilon_L - \epsilon_R \tag{2.4}$$

 ε_L : molar extinction coefficient of left-handed circular polarized light

1

 ε_R : molar extinction coefficient of right-handed circular polarized light

- *c*: molar concentration (mol/L)
- *l*: pathlength (cm)

For most instruments, the CD values are reported in degrees of ellipticity (θ). The following equation shows the conversion from ΔA to θ :

$$\theta(degrees) = \Delta A(\frac{\ln 10}{4})(\frac{180}{\pi})$$
(2.5)
Since the measured CD is not only a molecular property, but also depends on sample concentration, temperature and chemical environment, a corrected CD unit, known as molar ellipticity ($[\theta]$), is often used.

$$[\theta] = 3298.2\Delta\epsilon \tag{2.6}$$

Circular dichroism (CD) spectroscopy measures CD of molecules as a function of wavelength. CD spectroscopy has been widely used to study large biological molecules, like DNA, peptides and proteins.⁽¹¹⁰⁾ Due to its high sensitivity to protein and peptide secondary structural changes, CD spectroscopy can also monitor the protein and peptide structural changes as a function of temperature or time. CD spectra can be assigned to recognizable structural features of peptides and proteins in various absorption regions, particularly the far UV. Thus, CD spectroscopy is able to distinguish between α -helical, β -sheet and random coil secondary structures in peptides and proteins based on their distinct spectral band shapes in the far UV.

Various chromophores in proteins and peptides can absorb at several regions below 320 nm, including the peptide bond (below 240 nm), side chains of aromatic amino residues (around 260 nm to 320 nm) and weak and broad absorption for disulfide bonds (centered around 260 nm). For peptide bond region, two electronic transitions can be characterized, one is a $n \rightarrow \pi^*$ transition around 220 nm with a weak and broad absorption, the other is a more intense absorption for the $\pi \rightarrow \pi^*$ transition around 190 nm.⁽¹¹⁰⁾ In many protein folding studies, polypeptides are good models for studying conformational change based on their well-defined secondary structures. Figure 2.3 shows the CD spectra of poly-L-lysine (PLL) in different secondary structures.⁽¹¹¹⁾ The α -helical secondary structure gives rise to a deep negative band at 222 nm representing the

 $n \rightarrow \pi^*$ transition and a negative and positive couplet band at 208 nm and 192 nm for $\pi \rightarrow \pi^*$ transition, yielding the characteristic double negative with a positive component in the far UV. In contrast, β -sheet secondary structure is less well-defined, but normally has a weak and broad negative band at 216 nm and a more intense positive band around 195 nm. Random coil (unordered structure) can be identified by a deep negative band below 200 nm.

CD spectra can be used to estimate the protein secondary structure composition by processing with several different algorithms. These algorithms allow a quantitative analysis of CD spectra of proteins based on that of model proteins from a database, whose structures are well-defined by X-ray crystallography.⁽¹¹²⁾ The algorithms used for CD spectral analysis includes CONTIN, CDSSTR, VARSLC, K2d and SELCON.⁽¹¹³⁻¹¹⁶⁾ The CD Pro software package by Sreerama and Woody,⁽¹¹⁷⁾ that includes CONTIN⁽¹¹⁸⁾, CDSSTR⁽¹¹⁵⁾ and SELCON⁽¹¹³⁾, is available online for CD spectral analysis of soluble proteins. Additionally, further development of these computational algorithms provides the possibility to study the secondary structure of insoluble membrane proteins, whose experimental CD spectra are hard to acquire due to poor solubility and interference from membrane components.⁽¹¹⁹⁾

Another application of CD spectroscopy is the induced CD of achiral chromophores interacting with chiral substrates. Although some molecules have no inherent chirality, they can acquire measurable CD by coupling the chromophore to a chiral molecule or surface (macrostructure), for example, bound in asymmetric environment.⁽¹²⁰⁾ Thioflavin T (ThT) has induced circular dichroism (ICD) upon binding with amyloid fibrils through an extrinsic Cotton effect.⁽¹²¹⁾



Figure 2.3 CD spectra of protein secondary structures represented by poly-L-lysine at various pH and temperature conditions. Reprinted with permission from "Computed Circular Dichroism Spectra for the Evaluation of Protein Conformation", Greenfield, N. J. and Fasman, G. D., *Biochemistry* 1969, 8, 4108-4116. Copyright 1969 American Chemical Society.

2.4.2 ECD Instrumentation

For the work in this thesis, CD spectra were collected on a J-810 spectropolarimeter (JASCO, Inc.). Light from the light source passes through a set of mirrors, slits and two prisms, then becomes plane polarized. After that, the ordinary ray is focused by a lens and passed through a filter to the modulator (PEM), which alternately phase retards the light into mostly left or right-handed circular polarization states. The circularly polarized light then passes to the sample compartment and is detected by photomultiplier (PMT). Thermal variation of samples for CD spectra was accomplished using a thermoelectric controller accessory (CDF-426S/15, Jasco) controlled by the CD software. The CD instrument has been frequently calibrated by controlling for the intensity scale of (+)-camphor-10-sulfonic acid at 290 nm.⁽¹²²⁾

2.4.3 ECD Experimental Protocols

The protein solution concentration of ~ 0.2 mg/ml is used for the CD measurement. The CD spectra were obtained by averaging 8 scans with 50 nm/min scan speed, bandwidth of 1 mm and response time of 2 sec. The samples were measured in quartz cuvettes with different path lengths including 1 mm and 0.5 mm depending on sample conditions and experimental requirements. The variable temperature experiments were done by measuring complete CD spectra in 5 $\$ steps from 5 to 90 $\$ under the control of thermoelectric accessory (CDF-426S/15, Jasco) for both sample and background. The final spectra were obtained by subtracting background spectra from the corresponding sample spectra. CD data are presented in terms of molar ellipticity per residue ([θ]) according to the following equation:

$$[\theta] = \frac{\theta_{obs}}{10 \times c \times l \times n} \tag{2.7}$$

 $[\theta]$: molar ellipticity (deg cm² dmol⁻¹ residue⁻¹)

 θ_{obs} : CD signal, observed ellipticity (mdeg)

c: concentration (mol/l), which can be converted to g/l (mg/ml) by multiplying by the molar mass (g/mol) of protein or peptide

l: path length (cm)

n: number of residues in the protein

The fractional secondary structure for proteins was estimated from the CD spectra by use of CDPro software package.⁽¹¹⁷⁾

2.5 Infrared Spectroscopy

2.5.1 Principle of IR

Although ECD is a dominant technique for secondary structural study of proteins and peptides, it often needs bandshape-recognition-based algorithms to analyze unresolved overlapped ECD band shapes resulting from different conformations, which can be a problem.⁽¹²³⁾ By contrast, the IR technique provides only single-signed spectral bandshapes, but these are narrower and their relative frequencies can be assigned to characteristic vibrational modes of different bond types in the molecules. Therefore, Fourier transform infrared spectra (FT-IR) frequency shifts have been used for determining secondary structures for peptides and proteins. Unlike ECD, which is only appropriate for soluble samples, IR can measure samples in different conditions, including in solution, in hydrophobic lipid films or vesicles (membrane-like environment) or even in the solid state. In the latter case, a useful surface or thin-layer sensitive technique of attenuated total reflectance (ATR) has been used.⁽¹²⁴⁾ IR can be employed to study secondary structure, conformational transitions and orientation of insoluble biological molecules, especially membrane proteins.⁽¹²⁵⁾ Additionally, polarized ATR-FTIR can provide useful information about relative orientation between protein components and the membrane.⁽¹²⁶⁾

Based on the various vibrational normal modes of the amide group, nine amide bands can be assigned in IR spectra. The characteristic peptide group frequencies are shown in Table 2.1. Among these bands, three vibrational modes (amide I, II and III), as diagrammed in Figure 2.4, are commonly detected by IR for use in peptide and protein secondary structural studies.

The amide I vibrational mode has been most widely used to identify protein secondary structures. It is dominated by C=O stretching (~80%) along with a small contribution from C-N stretching motion (~20%). The amide II mode contains both N-H in-plane bending and C-N stretching, but the spectral band is very sensitive to solvent conditions. A large band shift (~ 100 cm⁻¹) can arise from changing the solvent from H₂O to D₂O due to the H/D exchange of the amide N-H. Although amide III band is very useful in Raman spectroscopy, it is less valuable in IR because of its weak absorption. Focusing on the amide I frequency, different secondary structures of protein can be identified, as shown in Table 2.2. For the α -helical conformation, the amide I band is in the range of 1657-1648 cm⁻¹, while protein with antiparallel β -sheet structure absorbs at a lower frequency, ~1630 cm⁻¹, often with a weak shoulder at higher frequency (~1680 -1690 cm⁻¹) and protein with parallel β -sheet structure absorbs at ~ 1620 cm⁻¹.

Mode	Frequency (cm ⁻¹)	Vibrational mode
А	~3300	NUL stratching in assessments with smile II scontone
В	~3100	INH stretching in resonance with amide II overtone
Ι	1700-1600	C'=O stretching with CN stretching and NH bending
II	1580-1480	C'N in-plane stretching, NH bending
III	1300-1229	C'N in-plane stretching, NH bending
IV	767-625	O=C'-N bending
V	800-640	NH out-of-plane bending
VI	606-537	C'=O out-of-plane bending
VII	~200	NH, C'=O, CN torsions

 Table 2.1 Characteristic IR Bands of Protein and Peptide Amide Group

Susi, H. (1972) Methods Enzymol. 26, 455.



Figure 2.4 Diagram of three protein amide vibrational modes (I, II, III).

Frequency (cm ⁻¹)	Assigned structure
1695-1670	Intermolecular β -structure (parallel β -sheet)
1690-1680	Intramolecular β -structure (antiparallel β -sheet)
1670-1660	3_{10} helix
1657-1648	a-helix
1645-1640	Random coil
1640-1630	Intramolecular β -structure (antiparallel β -sheet)
1625-1610	Intermolecular β -structure (parallel β -sheet)

Table 2.2 The characteristic amide I band frequency of protein secondary structure

The IR spectra of protein secondary structures, including α -helical, β -sheet and random coil are shown in Figure 2.5. Some polypeptides are good models for secondary structural study, since they can adopt variable structures in different conditions. For example, poly-L-lysine (PLL) can show α -helical, β -sheet or random coil conformations at different pHs and temperatures. ⁽¹²⁷⁾

2.5.2 IR Instrumentation and Experimental Protocols

In this thesis work, a Vertex 80 FTIR spectrometer (Bruker, MA) with deuterated triglycine sulfate (DTGS) detector was used to determine the secondary structure of proteins and peptides. The variable temperature experiments were realized by flow from a HAAKE DC50/K20 water bath under control of the spectrometer software. The ATR-FTIR experiments were conducted on the spectrometer with a MIRacle accessory (PIKE Technologies, Inc., Madison, WI), which incorporates a single-bounce diamond crystal ATR plate. If desired, polarization was obtained by insertion of a grid polarizer (Au on ZnSe substrate) in the beam before the ATR apparatus.

For FTIR, water solvent is a problem because its H-O-H bending can strongly interfere with the amide I band of protein and peptides. For samples in H₂O-based solution, FTIR measurement requires very high concentrations (as high as 60-100 mg/ml) to get high S/N absorbance and a very short path length (6 - 8 μ m) to eliminate the interference. Highly concentrated protein or peptide samples in the aqueous solution were placed into the commercial CaF₂ Bio cell (BioTools, Inc) consisting of two windows, one grooved down to provide an 8 μ m path length. Alternatively, D₂O can be used as a solvent instead of H₂O to avoid this problem because the D₂O bending mode is shifted to lower frequency (~ 1000 cm⁻¹).



Figure 2.5 FTIR spectra of proteins with primarily one type of secondary structure in H₂O. Amide I and II FTIR region of dominantly α -helix (myoglobin), β -sheet (Immunoglobulin) and random coil (α -casin) is shown. (Keiderling group archived data)

Samples dissolved in the deuterated solvent were placed in a sealed homemade IR cell consisting of two CaF₂ windows separated by a Teflon spacer of either 50 μ m or 100 μ m path length, depending on sample concentration. All the spectra were obtained using 4 cm⁻¹ resolution and averaging of 512 scans. Both sample and solvent were measured under the same condition and the solvent spectra were subtracted from corresponding sample spectra with a variable factor (in FTIR software) to get reasonable flat baseline. The water vapor spectrum was obtained by measuring the empty sample compartment with and without purging under the same conditions as sample and solvent and was variably subtracted from sample and solvent spectra to make them smooth. The final spectra were baseline and water vapor corrected. For the ATR-FTIR measurement, a 12 μ l sample was pipetted onto the crystal plate and dried by a gentle stream of nitrogen gas to form a multilayer film.

2.6 Vibrational Circular Dichroism

2.6.1 Overview

Vibrational circular dichroism (VCD) is a measurement of the difference in absorption of left-handed and right-handed circularly polarized light ($\Delta A = A_L - A_R$) by molecular vibrational transitions in the infrared (IR) region.⁽¹²⁸⁻¹³⁰⁾ The differential absorption intensity (ΔA) arises from the chiral interactions of molecular bonds in an asymmetric environment. During its early development, the VCD was used to measure a variety of small chiral organic compounds.⁽¹²⁸⁻¹³⁰⁾ Because the characteristic configurations of biopolymers can result typical spectral pattern, VCD measurements were then made on biological molecules, including proteins, peptides and nucleic acids (DNA and RNA).⁽¹³¹⁻¹³³⁾ Recently, VCD spectroscopy has been reported as an insightful approach for the study of amyloid fibrils, whose highly ordered supramolecular structure can enhance the VCD intensity.⁽¹³⁴⁻¹³⁶⁾

2.6.2 Principles and Applications of VCD

VCD theory is based on a coupling of changes of both electric and magnetic dipole transition moments. Calculation of the electric dipole moment is easier than for ECD because only the ground electronic state is involved, however, calculation for the magnetic dipole moment is more complex.⁽¹³⁷⁾ The VCD magnitude is dependent on the rotational strength, R_{01} , which can be expressed as the dot (scalar) product of the electric and magnetic dipole transition moments.⁽¹³⁸⁾

VCD can be used to determine the dominant secondary structure of proteins and peptides much as with IR or ECD. It requires relatively high concentration of protein or peptides in aqueous solutions to obtain enough absorbance for good signal-to-noise (S/N) VCD spectra. To acquire good S/N amide I VCD for peptide and protein in H₂O, very high concentrations are used, and short enough pathlengths are needed to obtain sufficient transmission. Alternatively, D₂O-based solvents can be used with low concentrations and longer pathlengths, so peptide and protein VCD are often focused on the amide I' vibration (C=O stretch) mode. The amide II' (N-D bending and C-N stretching) is also measurable, but the shift caused by H-D exchange makes it difficult to characterize or relate to the amide II (in H₂O). However, after H-D exchange, the amide A' (N-D stretch) and III' vibrations are not detectable due to solvent interference.⁽¹³⁹⁾ The secondary structures of peptides and proteins have characteristic VCD spectral sign patterns in the amide I' region, as shown in Figure 2.6.⁽¹³⁹⁾



Figure 2.6 VCD monitored secondary structures of model polypeptide. VCD and IR amide I' bands of deuterated poly-L-lysine peptide (in D2O) at (a) high pH and low temperature (α -helix, mixed with some β -sheet structures), (b) high pH after heating (β -sheet, aggregates) and (c) neutral pH (random coil). Adapted with permission from "Conformational Studies of Biopolymers, Peptides, Proteins, and Nucleic Acids. A Role for Vibrational Circular Dichroism", Keiderling, T. A., and Lakhani, A., *Comprehensive Chiroptical Spectroscopy*, John Wiley & Sons, Inc., 2012. Copyright (2012) John Wiley & Sons, Inc.

The right-handed α -helical peptide has a positive couplet (+ and -, from low to high frequency) amide I VCD but in D₂O a second negative at low frequency appears giving the amide I' a (-+-) pattern. The random coil form has an oppositely signed VCD spectrum from α -helical with an intense negative couplet amide I' band centered at ~ 1650 cm⁻¹. In the aqueous solution, peptide and protein β -sheet structures only yield weak amide I' VCD. However, aggregated polypeptides and proteins can give rise to an enhanced VCD with a strong couplet shape, which is correlated with macromolecular morphology of biopolymers and not to the secondary structure directly. ^(136, 140)

2.6.3 VCD Instrumentation

Two kinds of VCD instruments are in use: dispersive and Fourier transform. Dispersive VCD uses a grating monochromator, while FT-VCD uses a Michelson interferometer. In this thesis, a homebuilt dispersive VCD was used.⁽¹⁴¹⁾ The light from argon gas protected carbon rod light source is collected by a spherical mirror, focused onto a slit after being modulated at 150 Hz by a mechanical chopper. After the f/4 monochromator, the output light passes through a long-wave pass filter (to eliminate higher order diffraction) into the sample compartment. The IR beam is focused by a BaF₂ lens and passes through a linear polarizer and a photoelastic modulator (PEM) to produce circularly polarized light. The light passing through the sample is refocused by another BaF₂ lens onto a liquid-nitrogencooled MCT detector. The transmission and polarization modulation signals are digitized and normalized using the controlling computer.⁽¹⁴¹⁾Differential absorbance (ΔA) is obtained according to the following equation:

$$\frac{V_M}{V_T} = (1.15\Delta A) J_1(\alpha_0) g_1$$
(2.8)

 $J_1(\alpha_0)$: the first-order Bessel function at α_0 (peak retardation)

g₁: the overall instrument gain factor, can be corrected by calibration

V_M: polarization modulation signal

V_T: transmission modulation signal

The instrument is calibrated using a birefringent plate (CdS multiple waveplate) and polarizer pair in the sample compartment, which result in sine wave-like signal used as a calibration curve. The calibration factor ($J_1(\alpha_0)$), determined from the absolute values of the midpoint of zero-crossings in calibration curve, and gain factor (g_1) are needed to covert to the raw VCD spectrum.

2.6.4 VCD Experimental Protocols

The VCD detected vibrational transition is much weaker than the ECD detected electronic transition, thus in order to improve S/N, soluble protein and peptide samples with high concentration (20 - 40 mg/ml) are required for the VCD measurement. The same samples measured for both IR and VCD spectra are placed in the IR cell with desired pathlength. The amide I' IR absorbance should be controlled at ~ 0.5 to acquire an optimized S/N. For aggregated protein samples, the absorbance can be much lower, since it has giant VCD signal induced by the highly ordered supramolecular structure. Low absorbance helps minimize polarization and absorption artifacts in the VCD spectrum.⁽¹⁴¹⁾ For samples in water-based solvents, higher sample concentration (60-100 mg/ml) and a CaF₂ Bio cell (BioTool, Inc) with path length of ~ 8 μ m are used. The spectra were recorded at ~13 cm⁻¹ resolution as an average of 8 scans for samples in H₂O-based solvents over the amide I' vibrational range and 4 scans for samples in H₂O-based solvents over the combined amide I and II range. The baseline correction is done by

alignment and stabilization of the instrument resulting in a flat baseline after subtraction of the background spectrum from corresponding sample spectrum.

2.7 UV-vis AND Fluorescence Spectroscopy

2.7.1 UV-vis Experiment

The Cary 300 Bio UV-vis spectrometer (Varian, Inc.) was used in this thesis work. The absorbance of protein samples were measured in a quartz cuvette of 1 mm pathlength, and the concentrations were normally determined from absorbance at 280 nm using extinction coefficient of aromatic residues according to Beer's law.

2.7.2 Fluorescence Spectroscopy

Fluorescence spectroscopy is a very common technique in protein and peptide study, it can probe the fluorophore residues (Trp, Tyr) in proteins and peptides, dye molecules or fluorophores used to label the proteins. Due to its high sensitivity, it has been widely used to monitor the interaction of protein with other proteins,⁽¹⁴²⁾ with small ligands and membranes in protein folding and conformational transition studies.

Samples were placed in 1 mm path length quartz cuvette (dual pathlength, 1×10 mm with 4 polished windows) and were measured on a Fluoromax-3 spectrofluorimeter (Jobin-Yvon Inc., NJ). For the Trp fluorescence measurement, the excitation wavelength was 295 nm and the emission spectrum was scanned from 310 to 450 nm with a slit width of 5 nm. For the dye leakage experiments, the excitation and emission wavelength for fluorescein were 490 nm and 520 nm, respectively. In all the experiments, the background spectra of buffer or lipid were measured as the same manner as for the samples, and subtracted to obtain a final result.

2.8 Transmission Electron Microscopy

Electron microscopy has been used to monitor the morphology of aggregates of biomolecules in the amyloid fibril study. The TEM work in this thesis was conducted by Linda Juarez in the UIC RRC facility on a JEM 1220 electron microscope instrument (JEOL Co., Japan), with an operating voltage of 80 kV and Gatan Es 1000W 11 MP CCD camera. 15 μ l of diluted protein fibril solution was settled onto carbon-coated 400-mesh copper grids for 1 min, and then the excess was removed with filter paper. A 2 wt% of uranyl acetate negative staining solution was applied to stain the samples for 2 min. After being dried carefully to remove the excess sample and stains, the grids were ready for the TEM imaging.

Equilibrium and Dynamic Spectroscopic Studies of the Interaction of Monomeric β-Lactoglobulin with Lipid Vesicles at Low pH

The work in this chapter was published, "Equilibrium and Dynamic Spectroscopic Studies of the Interaction of Monomeric β -Lactoglobulin with Lipid Vesicles at Low pH", Ge Zhang and Timothy A. Keiderling, *Biochemistry*, **2014**, *53*, 3079-3087.

3.1 Overview

Protein folding and misfolding have been studied on many levels, one aspect of which involves interaction between proteins and membranes. The genome is coded for a great number of membrane-bound or active proteins. Furthermore, many extra cellular proteins interact with membranes and/or membrane proteins as part of their function, important examples of which are signaling and transport processes. Optical spectroscopic methods offer useful tools for study of complex protein-membrane systems and yield global, or averaged, structural data, which can be useful for monitoring folding changes and conformational equilibria. Optical spectra also can track dynamics due to their relatively fast time response.

Bovine β -lactoglobulin (β LG), a major component of cow's milk, is a member of the lipocalin or lipocalycin family.⁽¹¹⁴³⁾ In its native state, β LG is a soluble, globular protein with a predominantly β -sheet structure consisting of one major α -helix and a β -barrel of eight continuous antiparallel β -strands shaped into a flattened cone or calyx.^(52, 62) This can provide a pocket for binding fatty acids and other nonpolar molecules, but the prime biological function of β LG remains unknown. ⁽¹⁴⁴⁾At physiological pH values, a ninth β -strand forms H-bonds to the like strand in another β LG, creating a native state dimer, which dissociates to a monomer form below pH ~3,⁽⁵³⁾ and retains this monomer native

conformation even at pH values as low as 2.^(145, 146) The monomer and dimer share the same folded secondary structure.⁽⁵⁰⁾ Although the β LG secondary structure consists mainly of β -sheet components in its native state, structure prediction algorithms suggest that various segments of its sequence have a high propensity to adopt helical structures.⁽¹⁴⁷⁾ Non-native partially folded structure was observed during an early stage of β LG folding as shown by kinetics studies detecting an α -helical intermediate form in refolding β LG from a denatured, disordered form (random coil).^(74, 148) It has been shown that native state folding of β LG provides a good model for studying the mechanism of an α -helix to β -sheet (α - β) transition in proteins,^(74, 149) which can be an important mechanistic step for understanding the folding of a number of protein molecules, including misfolding related to amyloid diseases.⁽¹⁵⁰⁻¹⁵³⁾ On the other hand, equilibrium studies have shown that native state β LG unfolds undergoing a β -sheet to α -helix transition when transferred to solutions containing alcohols,⁽¹⁵⁴⁾ ionic surfactants,⁽¹⁵⁵⁾ and lipids.⁽¹⁵⁶⁾

Previous β LG-lipid interaction studies in the Keiderling group have shown that anionic lipid vesicles and anionic surfactant micelles can induce such a β -to- α conformational change in dimeric β LG at neutral or at somewhat reduced pH (4.6) and that the extent of the transformation depended on concentration and pH.⁽⁸⁰⁻⁸²⁾ It was demonstrated that electrostatic interaction between the negative lipid and positive protein was a prerequisite for this conformational change but also that hydrophobic interaction with the lipid bilayer played a secondary role in developing the final conformation. These studies also demonstrated that the helices, at least in part, insert into the membrane and lead to vesicle leakage at neutral pH. The resulting proposed interaction mechanism was a multistep process in which the extent of β LG interaction with the lipid vesicles was shown to depend on electrostatics as well as lipid packing or phase characteristics.⁽⁸⁰⁾

In this study, we turn from the native state dimer to address the equilibrium and kinetic aspects of interaction of the monomer form at low pH with lipid vesicles. Our results show an entirely different process, which depends primarily on hydrophobic interaction in studies of β LG interaction with zwitterionic lipids, as exemplified by distearoylphosphatidylcholine (DSPC), and results with other lipids will be also discussed. Lowering the pH in order to stabilize the monomer β LG form alters the nature of the lipid vesicles, such that the previously studied, negatively charged phosphatidylglycerol (PG) lipids, which have the pKa values in the range of $3\sim 3.5$,⁽¹⁵⁷⁾ become protonated. These were found to not induce a conformational change in β LG at pH 2.6. By contrast, the zwitterionic (PC) lipids, a main component of eukaryotic membranes, do lead to β LG structural change at low pH.

While PC lipids are affected by low pH, only a small fraction of their phosphate groups become protonated at pH 2.5.⁽¹⁵⁸⁾ The lipid phase transition is also shifted by varying the pH value.⁽¹⁵⁸⁾ Thus these monomeric studies effectively eliminate electrostatics as a driving force and let us focus on the role of hydrophobic forces in the protein-lipid interaction. Recently, it has been reported that the membrane binding of proteins with lipid vesicles can have a strong dependence on curvature when electrostatic forces are reduced, under conditions in which the interaction will be predominately driven by a hydrophobic mechanism.⁽¹⁵⁹⁾ These previous studies showed that the weaker the electrostatic interaction, the greater the dependence on hydrophobicity and membrane curvature.⁽¹⁵⁹⁾ All of these aspects are important for the monomer β LG-lipid interaction,

so that the previously reported charge driven β LG-lipid interaction mechanism at neutral pH⁽⁸⁰⁻⁸²⁾ is changed for the interaction between monomer β LG and zwitterionic lipids.

One hypothesis is that interaction of monomer β LG and zwitterionic lipid SUVs at low pH will be mainly driven by hydrophobic effects and the previously dominant electrostatic interactions at higher pH will be largely eliminated. Varying the lipid acyl chains for these low pH studies actually has dramatic effects on the interaction, in contrast to our experiences with previous studies at higher pH.⁽⁸⁰⁻⁸²⁾ In this case, the simplest results were obtained with PC lipids, mainly with DSPC, which are the focus of this chapter. The kinetics of these interactions were much slower than seen before with negatively charged lipids at higher pHs, so that the rates of secondary and tertiary structure change could be monitored conventionally by repeated scans of the CD and fluorescence spectra measured over the accessible spectral regions.

3.2 Materials and Methods

 β -lactoglobulin A from bovine milk (Sigma-Aldrich, L 7880, USA) was used without further purification. 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1, 2dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1, 2-dioleoyl-*sn*-glycero-3phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-di-(9,10-dibromo)stearoyl-*sn*-glycero-3-phosphocholine (Br-DSPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The saturated DSPC and DMPC lipids, has two 18-hydrocarbon chains and two 14-hydrocarbon chains, respectively. DOPC has two unsaturated 18-hydrocarbon chains. POPC has one saturated 16-hydrocarbon chain and one unsaturated 18-hydrocarbon chain. Organic solvents (methanol and chloroform, spectral grade) and sodium phosphate (Analytical grade) were purchased from Sigma. Deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc.

The lipid vesicles (SUV and LUV) were prepared in 20 mM phosphate buffer (pH 2.6) according to the protocols described in Chapter 2. For CD measurement, the protein concentrations were 0.1 mg/ml and 0.2 mg/ml while lipid concentrations were varied from 0 to 12 mM. The fractional secondary structure for β LG in the absence and presence of lipid vesicles was estimated from the CD spectra by use of the SELCON 3 method, which is part of the CDPro software package.⁽¹¹⁷⁾ For the fluorescence measurement, the excitation wavelength was 295 nm, and the emission spectrum was scanned from 300 to 450 nm with β LG concentration of 0.2 mg/ml (same as for CD). For the FTIR measurement, samples were prepared by dissolving β LG and DSPC SUVs separately in deuterated phosphate buffer (pD 2.6) and then mixing them to a concentration of ~ 1 mg/ml and 50 mM, respectively (chosen to match saturation conditions found in CD titration). The samples were incubated at 50 $^{\circ}$ C overnight and placed in a sealed homemade cell consisting of two CaF₂ windows separated by a 100 µm Teflon spacer. The spectral measurements for CD, fluorescence, FTIR and DLS were done followed the descried protocols in Chapter 2.

3.2.1 DLS Determined Sizes of Lipid Vesicles (SUV and LUV) Under Different Temperatures and pH Values

DLS measurements indicated the LUVs had a radius of ~60-70 nm. Both the SUV and LUV sizes were relatively stable up to 50 $^{\circ}$ C, more so at low pH, due to their higher temperature phase transition, but the SUVs increased in size at higher temperatures (~65 $^{\circ}$ C) to be equivalent to LUVs, shown in Figure 3.1.



Figure 3.1 Dynamic light scattering determined DSPC vesicle sizes for SUVs (pH 2.6, in black and pH 6.8, in green) and LUVs (pH 2.6, in red) at 25 $\,^{\circ}$ C, 50 $\,^{\circ}$ C and 65 $\,^{\circ}$ C. Error bars represent the mean + standard deviation of the radii of the DSPC lipid vesicles. At 25 $\,^{\circ}$ C, DSPC SUVs with different pH had similar sizes. While they behaved similarly as temperature increased, close to the normal DSPC lipid transition temperature (55 $\,^{\circ}$ C) at 50 $\,^{\circ}$ C the size of DSPC SUVs at pH 6.8 changed more than SUVs at pH 2.6, which could be due to the higher transition temperature of DSPC at lower pH. At 65 $\,^{\circ}$ C after the transition to the liquid crystalline phase, both SUVs expanded to be equivalent to the LUVs.

The required amount of β LG stock solution was slowly added to the aqueous lipid vesicle solution, and the solutions were mixed well. The β LG and vesicles were allowed to equilibrate at 50 °C overnight to form a stable protein-lipid complex for the equilibrium studies.

3.2.2 Fluorescence Quenching Experiment

Small aliquots of a 5 M acrylamide stock solution were added to the β LG in the absence or presence of lipid vesicles, and fluorescence emission spectra were recorded at an excitation wavelength of 295 nm. The data were analyzed according to the Stern-Volmer equation, $F_0/F = 1 + k_{sv}$ [Q], where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, and k_{sv} is the Stern-Volmer quenching constant.⁽¹⁶⁰⁾ Additional quenching experiments were done by adding Br-DSPC to the DSPC lipids in a 1:1 ratio. While quenching of the fluorescence was observed in this labeled, mixed lipid system, the effect was relatively small.

3.2.3 Polarized ATR-FTIR Measurements

Polarized attenuated total reflectance (ATR)-FTIR experiments were conducted on the Vertex 80 spectrometer (described in Chapter 2) with a MIRacle accessory (PIKE Technologies, Inc., Madison, WI), incorporating a single-bounce diamond crystal ATR plate. A 12 μ l sample of protein-lipid vesicle solution was pipetted onto the crystal plate and dried under a gentle stream of nitrogen gas to form a dry multilayer film. Spectra were recorded with both parallel (0[°]) and perpendicular (90[°]) polarization by use of a wire grid polarizer placed in front of the sample. The dichroic spectra were obtained by subtracting the weighted spectrum recorded with parallel polarized light from the spectrum recorded with perpendicular light, using a weighting coefficient, R_{iso}. For this experiment, R_{iso} was determined by integrating the area of the lipid carbonyl band at ~1740 cm⁻¹ from both 0° and 90° polarizations and ratioing them to give $R_{iso} = A_{90}/A_0$.^(80, 161, 162)

3.3 Results

3.3.1 Equilibrium and Kinetic Interaction of Monomeric β LG by DSPC Lipid Vesicles

CD and fluorescence were used to identify secondary and tertiary structural changes in monomeric β LG (the low pH form) upon interaction with zwitterionic lipid vesicles (DSPC). As shown in Figure 3.2a, the far-UV CD spectrum of β LG (0.2 mg/ml) in phosphate buffer at pH 2.6 (---) exhibits a broad, weak minimium around 216 nm, which is typical of β -sheet structure. With the addition of zwitterionic lipid DSPC (—), the CD spectra of β LG has a transition from a shape characteristic β -sheet to one typical of α helix, developing two, more intense minima at ~208 nm and 222 nm. The inset in Figure 3.2a shows the ellipticity change at 222 nm for 0.011 mM β LG (~0.2 mg/ml) as a function of DSPC concentration, and shows an increase in negative intensity that stabilizes at ~10 mM DSPC. The negative ellipticity at 222 nm of β LG at pH 2.6 increased almost linearly with addition of DSPC up to ~6 mM, and then slowly reached a plateau upon addition of more DSPC. Estimated secondary structure changes (determined using SELCON 3⁽¹¹⁷⁾ as a function of DSPC concentration are presented in Figure 3.2b. The DSPC-induced α -helix fraction of 0.2 mg/ml β LG reached a maximum value (~50%) when the DSPC concentration was more than 6 mM, which was correlated to a decrease of β -sheet fraction to a minimum value (~10%).



Figure 3.2 DSPC dependent conformational transition of β LG measured by far-UV CD (in molar ellipticity per residue unit). (a) Far-UV CD spectra of 0.2 mg/ml β LG in the absence (dash line, - - -) and presence of increasing concentrations of DSPC from 1 mM to 12 mM at pH 2.6 (solid lines, —). The inset shows the ellipticity change at 222 nm as a function of DSPC concentration. (b) Helical fractions (\blacksquare), sheet fractions (\bullet) and unordered fractions (\blacktriangle) are estimated as a function of DSPC concentration at pH 2.6 using SELCON 3 in the CDPro program. Solid lines indicate the trends of secondary structural change of each fraction as a function of DSPC concentration. Error bars represent standard deviations of the fractional secondary structure from three independent trials.

b

The far-UV CD spectrum of β LG at a lower concentration, 0.005 mM (0.1 mg/ml), was also measured with addition of DSPC at pH 2.6. The estimated secondary structure change for this lower β LG concentration, as shown in Figure 3.3, maximized at a lower DSPC concentration consistent with the DSPC-induced α -helix fraction reaching a maximum when the concentration ratio between β LG and DSPC was ~1:900 (mM: mM). The highest lipid concentrations resulted in considerable noise for $\lambda < 200$ nm, due to light loss, but the trends indicate a process consistent with the same sort of transition from native β -sheet structure to non-native α -helical structure at all concentrations studied.

This conversion of secondary structure is relatively slow at 50 °C (especially as compared to our previous neutral pH studies with negatively charged lipids).⁽⁸⁰⁻⁸²⁾ The CD shows a sharp drop in the first hour followed by a continued but slower change as illustrated in Figure 3.4. The transition shown, resulting from the interaction between β LG and SUVs from 10 mM DSPC at 50 °C, could be fit with a double exponential decay function,

$$\theta(t) = \theta_0 + \theta_1 \exp(-k_1 t) + \theta_2 \exp(-k_2 t)$$
(eq. 3.1)

that yielded fast and slow component rate constants, k_1 and k_2 , of 1.1 \pm 0.23 and 0.05 \pm 0.017 hr⁻¹, respectively.



Figure 3.3 Estimated Helical fractions (**•**), sheet fractions (**•**) and unordered fractions (**▲**) of the more dilute, 0.1 mg/ml, β LG as a function of DSPC concentration at pH 2.6 using SELCON 3 in the CDPro program. Error bars represent standard deviations of the fractional secondary structure from three independent trials.



Figure 3.4 CD detected kinetics for mixing SUVs of 10 mM DSPC with 0.2 mg/ml (0.011 mM) β LG at pH 2.6 monitored via change in the ellipticity at 222 nm for samples held at T = 50 °C. Solid line indicates fit by double exponential decay function (Equation 3.1, solid line), with k₁ = 1.1 ±0.23, k₂ = 0.05 ±0.017, R² = 0.98.

CD monitored dynamics at lower temperature, for β LG mixing with DSPC at 30 °C (see Figure 3.5), also showed a two-stage kinetic process with rates similar to but somewhat slower (k₁~0.93 ±0.35 hr⁻¹ and k₂~0.02 ±0.68 hr⁻¹) than what was observed at 50 °C (k₁~1.1 ±0.23 hr⁻¹ and k₂~0.05 ±0.017 hr⁻¹). However much less helical structure was induced at 30 °C (Figure 3.5); consequently the β LG- DSPC samples were incubated at 50 °C to induce more interaction and faster kinetics, in the results shown above (Figure 3.2 and Figure 3.4).

By contrast, if β LG is mixed with LUVs of DSPC at the same concentrations, relatively little CD change occurs, indicating only a minor increase in helicity (see Figure 3.6) which, by extension, means the interaction has a significant dependence on curvature of the bilayer.



Figure 3.5 Kinetic result for 10 mM DSPC mixing with 0.2 mg/ml β LG at pH 2.6 collected by monitoring the ellipticity at 222 nm at 30 °C, line indicates best fit by a double exponential decay function, with $k_1 = 0.93 \pm 0.35$ hr⁻¹, $k_2 = 0.02 \pm 0.68$ hr⁻¹ and R² = 0.83. Note: lower temperature results in less helix induction and slower kinetics than in Figure 3.4 (50°C).



Figure 3.6 Far-UV CD spectrum of 0.2 mg/ml β LG in the absence (solid line, —) and presence of 6 mM DSPC LUVs (dash dot line,-·-·) and 6 mM DSPC SUVs (dash line, ---) at pH 2.6, after incubation at 50 °C overnight.

As a probe of tertiary structure change, fluorescence spectra of β LG in various DSPC concentrations were measured (Figure 3.7a). Without DSPC, the fluorescence spectra of β LG at low pH (dot line) had a maximum (λ_{max}) at ~331 nm, indicating the emitting Trp residues were in a shielded hydrophobic environment. By adding various concentrations of DSPC SUV (solid lines, concentration increased from blue to red), the fluorescence intensity increased (as summarized in the inset of Figure 3.7a). This may be due to less quenching resulting from increased motional flexibility of Trp61, which in the native state may be quenched by the Cys66 – Cys160 disulfide bond.⁽¹⁶³⁾ However, the fluorescence band λ_{max} had a blue shift (to ~324 nm), with a residual shoulder at ~336 nm, which suggests that the protein does not fully unfold and the Trps do not become solvent exposed. This could be consistent with burial of at least one of the Trps (Trp61 or Trp19) in the lipid bilayer. As shown in Figure 3.7b, the blue shift of the maximum intensity also reached a maximum at ~6 mM of added DSPC (for 0.2 mg/ml β LG), suggesting the tertiary structural changes are correlated to the secondary structural changes.



Figure 3.7 DSPC-dependent tertiary structure change of β LG was measured by (a) fluorescence of 0.2 mg/ml β LG ($\lambda_{exc} = 295$ nm) in the absence (dot line, ...) and presence (solid lines, —) of increased concentrations (increased from blue to red) of DSPC lipid vesicles. The inset shows that intensities of λ_{max} in the normalized fluorescence increase as a function of lipid concentration. (b) The λ_{max} peak shift of the normalized fluorescence as a function of lipid concentrations.

b

This fluorescence-detected kinetic change for 0.2 mg/ml β LG mixing with 3 mM DSPC at 50 °C can be monitored in terms of change in the relative fluorescence intensity, as monitored at 331 nm and starting ~1.5 min after mixing (Figure 3.8), and can be fit with single exponential function,

$$F(t) = F_0 + a(1 - exp(-kt))$$
 (eq. 3.2)

that rises to a maximum with a rate constant, $k = 0.91 \pm 0.44$ hr⁻¹, which is consistent with the kinetics (~1.1 hr⁻¹) observed for the fast component of the CD intensity change. The parallel kinetics imply that the emitting Trp is transferred to a more hydrophobic environment when the secondary structure rearranges. However, there is also an initial burst step for both the frequency shift and intensity on initial change from β LG in buffer to β LG mixed with lipid (see Figure 3.9), which could be due to other fast structural changes of β LG or more likely to change in the solvent environment (index or scatter) following the fast mixing of protein and lipid.

While these results are consistent with our observations for β LG inserting in a lipid bilayer at higher pH, as controlled by the electrostatic driving force, we were not able to do the kind of leakage experiments as previously reported for higher pH.^(80, 81) However, we did measure acrylamide quenching, which showed the Trp residues to be relatively protected on binding to the SUVs (see Figure 3.10), consistent with results obtained for higher pH vesicles. Our efforts to use Br-DSPC lipids to quench Trp fluorescence and determine extent of Trp insertion were inconclusive.


Figure 3.8 The change in relative fluorescence intensity at 331 nm of 0.2 mg/ml β LG mixing with 3 mM DSPC SUV at 50 °C as a function of time, fit by a single exponential function (Equation 3.2, solid line), with k = 0.91 ±0.44, R² = 0.99.



Figure 3.9 (a) The fluorescence kinetic results of mixing 3 mM DSPC SUV with 0.2 mg/ml β LG at pH 2.6 at 50 °C, monitored by the λ_{max} peak shift of the normalized fluorescence as a function of time, where the solid line is the fit to a single exponential decay function, giving $k = 3.78 \pm 0.71$ hr⁻¹, R² = 0.92. (b) The relative fluorescence intensity change at 331 nm, the intensity of β LG in only buffer is the t = 0 initial point.

b



Figure 3.10 Stern-Volmer plots for acrylamide quenching of Trp fluorescence for 0.2 mg/ml β LG in the absence (• solid line, —) and presence of 6 mM DSPC at pH 2.6 (\blacktriangle , dash line ---).

3.3.2 Interaction of Monomeric *BLG* and Vesicles of Other Lipids at Low pH

In order to compare with DSPC, other zwitterionic lipids including DMPC, DOPC and POPC, were also used to interact with monomeric β LG at low pH. The interactions between monomeric β LG and these lipids are not spontaneous, and vesicles of these lipids only induced very little secondary structural change for low pH β LG even after long incubation times (over 40 hours) at temperatures above the phase transition temperatures of lipids. However, after brief sonication (10 min) of these mixtures of β LG and other zwitterionic lipid vesicles, significant secondary structural changes of β LG were observed, especially with DMPC and DOPC (Figure 3.11). According to the CD spectra (Figure 3.11a), DMPC SUV induced higher α -helical fractions for β LG and required less lipid concentration (6-8 mM) to reach the maximum β - α transition than did DOPC and POPC SUVs. CD spectra of β LG with concentrated DOPC and POPC SUVs have low signal-to-noise (Figure 3.11b and Figure 3.11c), due to the background effect of unsaturated lipids which contain a UV absorbing "C=C" in the acyl chains.

Fluorescence spectra of sonicated mixtures of β LG and vesicles of these three zwitterionic lipids show maximum bands at lower wavelength values than β LG without lipid vesicles at pH 2.6 (dash line, Figure 3.12), indicating the Trp residues went into a hydrophobic environment. β LG with 8 mM DMPC (solid line, Figure 3.12) and 12 mM DOPC SUVs (dot line, Figure 3.12) show the fluorescence maximum band at 325 nm with a residual shoulder at ~ 336 nm, which is consistent with fluorescence results for the β LG-DSPC interaction. However, the fluorescence maximum band of β LG with 12 mM POPC SUV present at 328 nm, indicates less tertiary structural change of β LG was induced by POPC.



Figure 3.11 Far-UV CD monitored three types of zwitterionic lipid vesicles induced conformational transition of monomeric β LG at low pH after 10 min sonication. Far-UV CD spectra of 0.2 mg/ml β LG in the absence (red solid lines) and presence of (a) DMPC SUV (2-8 mM), (b) DOPC SUV (2-12 mM, increased by 2 mM) and (c) POPC SUV (6 and 12 mM) at pH 2.6 (black solid lines). The insets show the ellipticity change at 222 nm as a function of DMPC, DOPC and POPC concentration, respectively.



Figure 3.12 Tertiary structural change of monomeric β LG with three types of zwitterionic lipid SUVs at low pH. Fluorescence of 0.2 mg/ml β LG ($\lambda_{exc} = 295$ nm) in the absence (dash line, - - -) and presence of 8 mM DMPC SUV (solid line), 12 mM DOPC SUV (dot line, ...) and 12 mM POPC SUV (dash-dot line, - -) at pH 2.6 after sonication.

3.3.3 Orientation of *β*LG Binding with Lipid Vesicles

Polarized ATR-FTIR was used to monitor the preferred orientations of β LG segments interacting with DSPC vesicles after they were deposited to form multilayer films on a surface. The differential polarized ATR-FTIR spectrum for β LG in DSPC (1:950 molar ratio) at pH 2.6 is shown in Figure 3.13. The difference spectrum $(90^{\circ} - 0^{\circ})$ was obtained using a weighting coefficient R_{iso} of 1.31. The progression bands in the region of 1350 cm⁻¹ to 1180 cm⁻¹ corresponding to positive difference peaks arise from wagging motions of the methylenes in the lipid acyl chains and the negative difference peak at 1465 cm⁻¹ is due to their CH₂ scissoring bands. Assuming the lipid chains were oriented perpendicular to the plate and these scissor mode vibrations are normal to the chain extension direction, the negative bands can be assumed to indicate dipole transitions oriented parallel to the surface of the membrane and positive bands indicate perpendicular orientation.⁽¹⁶⁴⁾ For the amide I region, the polarization difference spectra at the ATR plate for the β LG-DSPC complex film at pH 2.6 showed two weakly positive peaks at 1646 cm⁻¹ and 1633 cm⁻¹, which can be assigned to the β LG α -helix and β -sheet components, respectively. For the α -helix component, the positive peak at 1646 cm⁻¹ corresponds to a C=O stretching mode polarized parallel to the helix axis, and by this result, the helix axis appears to be primarily oriented perpendicular to the lipid membrane surface, which is consistent with insertion of at least a significant fraction of the helical segments into the membrane. For the β -sheet component, the C=O stretching vibration is perpendicular to the β -sheet strand direction, so that the positive amide I difference peak at 1633 cm⁻¹ indicates the β -strands are oriented more parallel to the bilayer surface and possibly lie on the membrane-solvent interface at pH 2.6.



Figure 3.13 Polarized ATR-FTIR difference spectra (90° - 0° polarization) of β LG in the DSPC bilayer with molar ratio 1:950 (top, black, —) and just DSPC (bottom, red, —) at pH 2.6.

3.3.4 Thermal Stability of the Lipid-*β*LG Complex

The thermal stability of β LG only and lipid-bound β LG complex at pH 2.6 as monitored by far-UV CD is shown in Figure 3.14. With increasing temperature, the ellipticity at 204 nm of β LG alone (**▲**) was initially stable and then sharply became more negative well above 70 °C, consistent with the protein undergoing a β -sheet to unordered transition (see Figure 3.14a and b for β LG and β LG-DSPC CD spectra, respectively). The transition did not reach a stable high temperature state, since the CD continued to change, as the temperature was increased to 90 °C. This indicates that the T_m of β LG at pH 2.6 is higher than that at neutral pH, which is ~68 °C based on our previous study.⁽⁸⁰⁾ By contrast, with increasing temperature, the ellipticity of the β LG-lipid complex at 222 nm (•) decreases, becoming less negative, and undergoes a two-stage transition. The result was fit with two sigmoidal functions having T_m values of 27 ± 3.6 °C, R² = 0.96 and ~67 ± 1.3 °C, R² = 0.97, respectively (fit is in Figure 3.15 with the corresponding spectra in Figure 3.14).⁽¹⁶⁵⁾

The thermal transition of β LG only and the lipid-bound β LG complex as monitored by FTIR at higher concentration was roughly consistent with the CD results (see Figure 3.16 for the spectra and Figure 3.17 for the variation). For β LG alone, the intensity for the β -sheet component (1633 cm⁻¹) (\blacktriangle) fluctuated with change in temperature but was relatively stable and then decreased sharply above 75 °C, consistent with the unfolding process monitored by CD. The FTIR of the α -helical component band (1644 cm⁻¹) for the β LG-DSPC complex showed a lower temperature transition than seen in CD with a T_m of 60 ± 2.1 °C, R² = 0.94, determined by fitting to a sigmoidal thermodynamic function.⁽¹⁶⁵⁾



Figure 3.14 (a) The thermal melting of 0.2 mg/ml β LG in buffer at pH 2.6 from 5 °C to 90 °C (blue to red), and (b) the thermal melting of the 10 mM DSPC : 0.2 mg/ml β LG complex at pH 2.6 from 5 °C to 90 °C, both as monitored by far-UV CD spectra.



Figure 3.15 Thermal stability measured by monitoring the far UV-CD ellipticity at 222 nm for 0.2 mg/ml β LG with 10 mM DSPC (•) and at 204 nm for the 0.2 mg/ml β LG in just buffer at pH 2.6 (\blacktriangle) (corresponding CD spectra are available in Figure 3.14a and b).

Based on the FTIR spectrum, the β LG-DSPC maintained broad peaks around 1640 cm⁻¹ indicating mixed β -sheet and α -helix components from 5 °C to 60 °C (Figure 3.16b, top) and developed a typical β -sheet component after the transition and maintained it between 65 °C to 75 °C (Figure 3.16b, bottom), but then it went through a second β - to unordered transition above 80 °C. Consistent with the thermal CD result, the decrease in the fraction of helical structure correlated with the thermal transition of DSPC lipid and may indicate a dissociation of the protein-lipid complex leading to refolding the more native structure at ~ 60 °C followed by unfolding process above 80 °C rather than being due to simply unfolding the protein while still associated with the lipid. It should also be noted that the SUVs grew in size, presumably due to fusion with other SUVs, to become equivalent to LUVs in size at 65 °C (see Figure 3.1), which is not preferred in the β LG-DSPC interaction (Figure 3.6).

The transition temperature for change in the ordering of the lipid bilayer in the vesicles was determined by monitoring the IR frequency for the CH₂ asymmetric and symmetric stretching modes of the aliphatic chains. For DSPC the asymmetric CH₂ mode frequency increases from 2917.6 cm⁻¹ to 2923.8 cm⁻¹, and the symmetric CH₂ mode increases from 2850.1 cm⁻¹ to 2853.4 cm⁻¹ as temperature is increased with a transition temperature of 61 ± 0.8 °C at low pH. After mixing with protein, the lipid transition did not change much, shifting to 64 ± 1.5 °C as monitored with the asymmetric CH₂ mode, and to 65 ± 0.7 °C for the symmetric mode. All the data were fit with a sigmoidal thermodynamic function (as shown in Figure 3.18).



Figure 3.16 (a) The thermal melting of 1 mg/ml β LG in buffer at pH 2.6 from 5°C to 95°C (blue to red), and (b) thermal melting of 50 mM DSPC and 1 mg/ml β LG complex at pH 2.6 from 5 °C to 60 °C (top) and 65 °C to 95 °C (bottom), both as monitored by FTIR. The arrows indicate the trends of peak shifts as a function of temperature.

b



Figure 3.17 Thermal stability measured by monitoring the FTIR absorbance intensity (•) at 1644 cm⁻¹ for 1 mg/ml β LG with 50 mM DSPC and (\blacktriangle) at 1633 cm⁻¹ for 1 mg/ml β LG in only buffer, both at pH 2.6 (FTIR spectra are in Figure 3.16).



Figure 3.18 Temperature dependence for 50 mM DSPC only (blue line, —, •) and the 1 mg/ml β LG : 50 mM DSPC complex (black line, —, **▲**) at pH 2.6 monitored by the FTIR maxima for DSPC aliphatic CH₂ stretching modes from 5 °C to 95 °C. Data shown for IR asymmetric stretching mode (top panel) and symmetric stretching mode (bottom panel) were fit by a sigmoidal thermodynamic function.

Since another zwitterionic lipid, DMPC SUV can significantly induce β - α transition of monomeric β LG at low pH with the help of sonication, the thermal stability of β LG-DMPC complex monitored by CD were also determined in the same manner as β LG-DSPC complex. The molar ellipticity of β LG-DMPC complex at 222 nm became less negative with increasing temperature, showing a two-stage transition with T_m value of 47 $\pm 0.8 \ \C$ (Figure 3.19). The FTIR intensity of the α -helical component band at 1642 cm⁻¹ of the β LG-DMPC complex decreased as a function of temperature, showing a two-stage transition with a lower T_m value of 32 $\pm 2.7 \ \C$ (Figure 3.20).

Similar to β LG-DSPC complex, the FTIR frequency for the CH₂ asymmetric and symmetric stretching modes of DMPC did not change much with and without protein. For DMPC only, the asymmetric CH₂ mode changed from 2918.9 cm⁻¹ to 2924.6 cm⁻¹ with a transition of 31.2 ± 1.2 °C and the symmetric CH₂ mode changed from 2850.6 cm⁻¹ to 2854.1 cm⁻¹ with a transition of 36.9 ± 1.3 °C (Figure 3.21, blue lines). For the β LG-DMPC complex, the transition of DMPC as monitored with the asymmetric CH₂ mode shifted to 35.9 ± 1.1 °C, and the transition for the symmetric mode is 37.0 ± 0.7 °C (Figure 3.21, black lines).



Figure 3.19 Thermal stability for 0.2 mg/ml β LG with 8 mM DMPC (•) at pH 2.6 measured by monitoring the ellipticity at 222 nm with far-UV CD. Line indicates fit to a sigmoidal thermodynamic function, yielding T_m = 47 ±0.8 °C, R² = 0.99.



Figure 3.20 Thermal stability for 3 mg/ml β LG with 114 mM DMPC (•) at pH 2.6 measured by monitoring the intensity at 1642 cm⁻¹ with FTIR. Line indicates fit to a sigmoidal thermodynamic function, yielding T_m = 32 ± 2.7 °C, R² = 0.96.



Figure 3.21 Temperature dependence of 114 mM DMPC only (blue line, —, •) and 3 mg/ml β LG and 114 mM DMPC complex (black line, —, **▲**) at pH 2.6 as monitored by the FTIR maxima for CH₂ stretching modes from 5 °C to 90 °C. Data for the IR asymmetric stretching mode (top panel) and symmetric stretching mode (bottom panel) were fitted by sigmoidal thermodynamic function.

3.4 Discussion

3.4.1 The Driving Force for the Association of β LG with DSPC

The interactions between proteins and lipids involve several steps: initial binding to the surface, induction or stabilization of a specific lipid-bound secondary structure, modulation of membrane biophysical properties by protein binding, and finally either partial or full insertion of the protein into the membrane bilayer.⁽¹⁶⁶⁾ This interaction is driven by the difference in stability of the protein in buffer as opposed to its stability when associated with the lipid vesicle, which offers a complex, non-homogeneous environment involving a charged or highly polar surface and a hydrophobic interior. The association of water-soluble proteins with lipids thus depends on the surface attraction due to overall electrostatic force,⁽¹⁶⁷⁾ stabilization by hydrophobic interaction,⁽¹⁶⁸⁾ or both.⁽¹⁶⁹⁾ The electrostatic forces will be determined by the relative charge of the lipid and protein and can be modified by pH, while the hydrophobic aspect can be enhanced by unfolding the protein to expose inner residues or by disrupting the surface to promote access to the core of the bilayer, which as recently shown, can be enhanced by the increased curvature in small vesicles (SUVs as compared to LUVs). (159) In this process, the protein structure may undergo dramatic changes, including a disruption of tertiary structure and/or a formation of specific lipid-bound secondary structure, both of which can depend on a change in the lipid physical state.

Previous studies from the Keiderling group showed that the main driving force of the β -to- α transition in dimeric β LG at pH 6.8 and pH 4.6 as induced by anionic lipid vesicles was due to electrostatic interaction, since zwitterionic lipids alone did not induce significant changes in the secondary and tertiary structure of β LG at those pH values.⁽⁸⁰⁻

⁸²⁾ Monomeric β LG at pH 2.6 has a net positive charge since it has a pI of 5.3, and zwitterionic lipid DSPC has only a small protonated fraction (~2.6% of the phosphate groups are reported to be protonated).⁽¹⁵⁸⁾ Our CD results showed a clear β - α transition upon addition of DSPC SUVs to monomeric β LG at low pH (Figure 3.2). The α -helical fraction increased to ~50% at the expense of the β -sheet fraction, which is consistent with previous results for the interaction of dimeric β LG with anionic lipids at higher pH.⁽⁸⁰⁾ By contrast the protonated anionic lipid DSPG at low pH did not induce a conformational change in β LG. In addition, the DSPC (as well as DMPC) SUVs at lipid gel phase can bind with β LG, but rapidly lost the β LG after undergoing a lipid phase transition (gel to liquid phase), and LUVs of DSPC did not significantly bind β LG or alter its secondary structure, as monitored by CD (Figure 3.6). The DSPC SUVs were shown to be stable in size, as measured by DLS, up to 50 °C, which is the temperature used for our binding measurements, but at 65 °C they fused and became equivalent in size to the LUVs (Figure 3.1), and during this process, β LG dissociated from protein-lipid complex.

The far-UV CD spectrum showed that relatively little α -helix was induced in β LG by DSPC LUVs (Figure 3.6), indicating a sensitivity of the interaction between monomeric β LG and DSPC lipid vesicles to curvature. This observation combined with the loss of binding after the lipid phase transition and fusion of the SUVs to form LUVs suggests the binding is dependent on curvature of the bilayer. The higher curvature of the SUVs can expose the core of the bilayer to interaction with hydrophobic residues, which, in the absence of electrostatic binding, can become the dominant interaction and can promote the insertion of the hydrophobic part of the protein into the bilayer, as recently demonstrated ⁽¹⁵⁹⁾

In comparison to previous studies of β LG-lipid vesicle interaction at neutral pH, ⁽⁸⁰⁻⁸²⁾ the kinetics as monitored by CD change show that monomeric β LG interaction with DSPC lipid at pH 2.6 and 50 °C had a much slower rate of structural change and required more concentrated lipids to achieve the final maximum helical structure (Figure 3.2 and Figure 3.4) than was the case for the neutral pH anionic lipids. However, the interaction between β LG and DSPC at 30 °C showed a slower kinetic, and less helical structure of protein was induced as monitored by CD (Figure 3.5). This temperature dependence of the degree of helicity gained suggests a dynamic aspect to the hydrophobic interaction, which could be due to increased exposure of the bilayer core correlated to motion of the head groups, or it could suggest that increased lipid dynamics closer to the lipid phase change favors insertion of helical segments.

The fluorescence intensity change with mixing β LG with DSPC had similar kinetic behavior as the CD fast component (Figure 3.8), but there was an initial fast mixing step in the deadtime (Figure 3.9). The overall kinetic change suggests that the change in solvent environment at 50 °C from an aqueous monomer low pH state to an α -helical DSPC membrane-bound state may cause β LG to first form an intermediate (molten globule state), in which the protein may be partially unfolded and expose its hydrophobic components, and then induced by the hydrophobic effect, it slowly inserts into the highly curved SUV lipid bilayers to develop more helical structure. A molten globule intermediate state has also been previously proposed to explain the lack of coincidence for mid-transition points of the secondary and tertiary structure changes in β LG.^(77, 170, 171)

Other zwitterionic lipids for example, DMPC, DOPC and POPC, can also induce a secondary structural change in low pH β LG, but vesicles of these lipids required

sonication to effect the protein-lipid interaction and promote structure change (Figure 3.11 and Figure 3.12). This suggests a more complex mechanism, probably one in which sonication causes incorporation of protein in the bilayer followed by unfolding and restructuring. The desolvation of the protein on mixing under sonication may overcome a kinetic barrier to protein-vesicle initial interaction. Since the protein hydrophobic clusters can be more easily exposed in a molten globule state, being in a lipid environment can stabilize hydrophobic residues in the restructuring process.⁽¹⁷²⁾ Given the loss of electrostatic driving force at low pH, the main interaction between the positively charged β LG and the zwitterionic lipid vesicles becomes hydrophobic. Thus the effectively mechanical mixing of lipid and protein provided by sonication, possibly partially unfolding the protein, while breaking and reforming the SUVs, could better expose the protein to the hydrophobic core of the lipid bilayer structure. In some sense the sonication of lipids has an even bigger advantage for promoting hydrophobic lipid-protein interaction than the increased access to the bilayer core proposed in the SUV vs. LUV comparison discussed above. Sonication was also used for β LG-DSPC SUVs interaction for comparison, and DSPC SUVs induced similar amount of α -helical fractions of β LG with and without sonication. The interactions between the monomeric β LG and zwitterionic lipids also depend on the character of the lipids, such as the length and saturation of the hydrocarbon chain and their effect on chain ordering and the temperature of the phase transition. DOPC and POPC, which have unsaturated hydrocarbon chains and lower temperature phase transitions, induced lower α -helical fractions than did the saturated lipids (Figure 3.11b and c).

3.4.2 Insertion and Orientation of β LG to Lipid Bilayers

Fluorescence can provide useful information about the solvent exposure or accessibility of tryptophan residues in the proteins or proteins associated with lipid vesicles. The λ_{max} blue shift (~9 nm) and reduced quenching of the fluorescence for βLG mixing with different DSPC concentrations (Figure 3.7) indicated the emitting tryptophan residues in β LG were shielded by insertion into the hydrophobic lipid bilayers. Stern-Volmer plots of the quenching of the Trp fluorescence in β LG with and without DSPC by added acrylamide showed that increasing the concentration of quencher resulted in a decrease in the fluorescence intensity in both media (Figure 3.10) This behavior indicates that the accessibility of the fluorophore did not change substantially through β LG expansion as induced by interaction with DSPC, which is consistent with the previous high pH, DMPG study.⁽⁸⁰⁾ The polarized ATR-FTIR results demonstrate that the α -helical components of the β LG-DSPC complex are preferentially perpendicular to the membrane surface and suggest that the residual β -sheet components are preferentially parallel to the membrane surface (Figure 3.13) This implies partial insertion of β LG into DSPC lipid bilayers, which is consistent with previous high pH results.⁽⁸⁰⁾ If the Trp residues were on the sequences which inserted, that would be consistent with our results. Studies of mutants that alter Trp residues to other hydrophobic amino acid residues (Ala, Val and Leu) could address this in the future.

3.4.3 Stability of *β*LG-DSPC Complex

FTIR can be used to study thermal transitions in both protein secondary structure and lipid vesicle structure.⁽¹⁷³⁾ The CD and FTIR thermal stability results showed the T_m of the β LG-DSPC complex was ~60-65 °C, at which temperatures the native monomeric

 β LG is stable (Figure 3.15 and Figure 3.17). However, coincident with the phase transition of the DSPC at pH 2.6 (Figure 3.1), the size of the DSPC SUVs at 65 °C increased to that characteristic of LUVs, presumably resulting in dissociation of the protein-DSPC complex. By contrast, the source of the initial thermal transition seen in the CD of the protein-lipid complex at ~27 °C is unclear. The thermal variations of FTIR spectra have fluctuations that may correspond to this transition, but they are too weak to be reliable.

The increase in the frequency of both CH₂ asymmetric and symmetric modes indicates increased flexibility of the lipid alkyl chains,⁽¹⁷³⁾ and is traditionally used to determine the phase transition, which is ~60-65 °C for DSPC at pH 2.6 (Figure 3.18). The binding of β LG to DSPC lipid vesicles does not significantly disturb the lipid phase transition equilibrium, which is consistent with previous conclusions that β LG does not affect the global organization or ordering of the vesicle, although it can insert into the hydrophobic lipid bilayers and cause leakage.^(80, 81)

Similarly, the β LG-DMPC complex had a transition at ~35-45 °C (Figure 3.19 and Figure 3.20), which is close to the DMPC phase transition (~30-35 °C) at pH 2.6 (Figure 3.21). Thus the thermal destabilization of β LG-lipid complex is correlated with the lipid bilayer phase transition, and indicates that the protein dissociates from the lipid bilayer as the lipid aliphatic chains increase in flexibility. This behavior is somewhat contrary to what is seen at high pH, where increased α -helical component was found for lipid bilayers in the higher temperature (liquid crystalline phase) as opposed to the lower temperature gel phase. At low pH, our results suggest the gel phase can trap the protein, presumably at least partly in the hydrophobic bilayers, but this trapping appears to

involve a barrier making the kinetics of the interaction much slower than found previously at higher pH. As the acyl chains gain flexibility at higher temperature (above the lipid phase transition), the lipid bilayer releases the protein, so potential faster kinetics of binding to a liquid crystal phase cannot be observed when only hydrophobic forces are at involved. At high pH, the protein-lipid complex is somewhat stable even at high temperature (higher than the lipid phase transition), which is presumably due to the added electrostatic attraction, which is not available at low pH. Thus the low pH monomer and neutral pH dimer have different mechanisms for both binding different lipid forms, zwitterionic vs. negatively charged, and also for dissociating from them.

3.5 Conclusion

Zwitterionic phospholipids can induce a high degree of non-native α -helical structure in monomeric β LG at low pH. The mechanisms of this interaction, the kinetic process of the conformational transition and the induction of stability for the lipid-induced complex are different from the previous high pH β LG-anionic lipid model. The monomeric protein may initially interact with the lipid to form an intermediate state (molten globule) between the native β -sheet dominant and lipid vesicle bound α -helical states. This interaction exposes hydrophobic protein residues to the lipid bilayer, and the large curvature of the DSPC SUVs that exposes the hydrophobic acyl tails can promote this interaction. The absorption and insertion of β LG into zwitterionic lipid bilayers at low pH appears to be driven by desolvation and consequent hydrophobic stabilization. As a consequence of a weaker driving force (at a distance) for interaction, the kinetics of the secondary and tertiary structural changes are relatively slow as compared to the electrostatically driven interaction at neutral pH. Once the protein is partially inserted into

the gel-phase lipid bilayer, β LG changes its structure, presumably due to the hydrophobic environment. The protein-lipid vesicle complex dissociates when the lipid undergoes a phase transition to a more fluid form at increased temperatures. This study may help to better understand potential biological functions of β LG, perhaps as an intracellular transporter of fatty acids. The molten globule intermediate state of β LG appears to be important in the low pH β LG-lipid interaction. It also been reported that molten globules are intermediates existing in the globular protein folding and unfolding process,⁽¹⁷⁴⁾ and the aggregation process of some proteins can be accelerated by transition through a molten globule state.^(172, 175, 176) Thus the study of interaction of β LG in the molten globule state may also provide useful information to the unfolding and aggregation processes of β LG.

4. DMSO Induced Destabilization and Disassembly of Various Structural Variants of Insulin Fibrils Monitored by Vibrational Circular Dichroism

This chapter contains research work submitted for publication, "DMSO Induced Destabilization and Disassembly of Various Structural Variants of Insulin Fibrils Monitored by Vibrational Circular Dichroism", Ge Zhang, Viktoria Babenko, Wojciech Dzwolak and Timothy A. Keiderling, *Biochemistry*, 2015.

4.1 Overview

The formation of amyloid fibrils is normally caused by non-native self-assembly of polypeptide chains to form cross- β aggregate structures,⁽¹⁷⁷⁻¹⁷⁹⁾ with few exceptions for biologically functional fibrils.^(180, 181) Numerous amyloid-related diseases, including diabetes type II, Alzheimer's, Creutzfeldt-Jakob, Huntington's and Parkinson's disease, have as pathological hallmarks the deposition of amyloid fibrils in tissues and organs, which at some point in the process (e.g. very early as pre-fibrillar aggregates) are toxic and result in structural and functional damage.⁽¹⁸²⁻¹⁸⁴⁾ Many proteins and polypeptides have been used for *in vitro* study of amyloid fibril formation. One of the model proteins is bovine insulin (structural details are described in Chapter 1, Figure 1.12), which can access partially or fully unfolded states under various environmental perturbations and adopt amyloid-like aggregates *in vitro* over a prolonged period.⁽¹⁸⁵⁻¹⁸⁷⁾ Like other amyloid fibrils, insulin fibril has a β -sheet core structure elongating along the fibril axis to form a twisted fibril structure, which can be observed by electron microscopy (TEM and SEM) and atomic force microscopy (AFM).⁽¹⁸⁸⁻¹⁹⁰⁾ This common cross- β core structural motif

suggests the possibility of a relationship between the β -sheet favored twist and higherorder chirality observed for such fibrils on the morphological level. ⁽¹⁹¹⁾

Thus, it is important to acquire more details about the chiroptical properties of amyloid fibrils using multiple spectroscopic techniques. Although Raman optical activity (ROA) can be used to study amyloidogenic intermediate states of some proteins, ⁽¹⁹²⁾ its application for amyloid fibril studies is still limited.⁽¹⁹³⁾ Far-UV circular dichroism (CD) has been widely used to probe secondary structure of soluble proteins, but poorly soluble amyloid fibril samples form micro particles which can give rise to distortion in CD spectra due to light scattering.⁽¹⁹⁴⁾ However, with care, CD can provide qualitative insight into the fibril assembly and chirality. Beyond this, CD can provide chirality information relevant to amyloid fibrils in an indirect way. Dzwolak et al. reported that thioflavin T (ThT), a dye with high affinity to amyloid fibril, can be used to probe the surface chirality of insulin fibrils by monitoring induced circular dichroism (ICD) detectable for ThT on its binding to fibrils.⁽¹⁹⁵⁾ They obtained insulin fibrils by agitating insulin solution at low pH, in the presence of salt within selected temperature ranges.⁽¹⁹⁶⁾ According to the ICD sign pattern at ~450 nm for ThT bound to these insulin fibrils, the resultant structures can be classified as either a +ICD fibril form (positive ICD sign) or a -ICD fibril form (negative ICD sign) according to the dominant species formed at a given temperature.^{(196,} ¹⁹⁷⁾ While high temperature favors one form (-ICD) and low temperature favors the other (+ICD), at intermediate temperatures (\sim 40 °C) either form can dominate in a selected, isolated solution sample. Thus both +/-ICD insulin fibrils can be prepared under the same conditions.

Vibrational circular dichroism (VCD) in the infrared range has been reported by Nafie, Lednev and co-workers to provide a useful method for determination of the macromorphological chirality of fibrils that develop an enhanced amide mode VCD.⁽¹³⁶⁾ Their extensive studies showed that insulin fibrils could give rise to VCD with an oppositely signed spectral pattern by varying the pH value during fibril growth.⁽¹³⁴⁾ Their preparation methods resulted in two dominant fibril types: "Normal form" (NF) was obtained at pH 2.4-3.2 and had a VCD sign pattern of "positive- negative-positive" over the amide I and II vibrational regions, while less intense, but "Reversed form" (RF) insulin fibrils were obtained at pH 1.1-2.1 and yielded a flipped VCD sign pattern.^(134, 135) Furthermore, NF and RF fibril forms were shown to have distinctive morphologies as observed by AFM and SEM, with NF having a left-handed twist and RF being more like a flat ribbon.^(134, 135) The RF form was reported to convert to an NF type by elevating the pH value during the fibrillation process, a change which can be monitored by VCD.⁽¹³⁴⁾ These authors concluded that the VCD spectral sign pattern for these fibril forms correlates with their AFM/SEM-determined supramolecular morphology and showed that this pattern occurred for several other protein-fibril aggregates.⁽¹⁹⁸⁾

The mechanism of amyloid fibril formation is complex and the detailed threedimensional structure of amyloid fibril is hard to determine due to its insolubility and noncrystallizable character. Since dimethyl sulfoxide (DMSO) can interfere with the protein hydrogen-bonded network leading to denaturation,⁽¹⁹⁹⁾ it has been used to solubilize and destabilize insulin fibrils.^(200, 201) Early FTIR and AFM studies showed that DMSO can convert amyloid fibril into soluble β -aggregates, and eventually a DMSO- solvated disordered (or random coil-like) form can be obtained in 90 wt% DMSO solution.⁽²⁰²⁾

In this study, VCD is utilized to probe the DMSO-induced disaggregation process of insulin fibrils and correlate their resulting VCD spectra with their TEM-defined supramolecular morphology. We used both +/-ICD insulin fibrils, that were formed at the same temperature with agitation and added salt, as well as NF/RF insulin fibrils, that were formed at different pH values but without salt or agitation, to compare the chiroptical behavior of insulin fibrils with different morphologies and formation histories under addition of DMSO. Under high concentrations of DMSO, all types of insulin fibrils can be mostly converted to a DMSO-solvated disordered structure, which is consistent with a previous FTIR study.⁽²⁰²⁾ However, an irreversible conversion from NF to RF insulin fibril (the opposite of the pH-induced change) in concentrated DMSO solution was observed with both VCD and TEM, but was not detectable with IR. These new results suggest that a relatively stable intermediate was formed during the disaggregation process, as will be discussed.

4.2 Materials and Methods

Insulin from bovine pancreas (Sigma-Aldrich, I6634) was used without further purification. Thioflavin T (ThT) was obtained from Sigma-Aldrich, and dimethyl sulfoxide (\geq 99.7%) and sodium chloride from Fisher Scientific. Deuterium oxide and deuterium chloride (20% w/w solution in D₂O) were purchased from Cambridge Isotope Laboratories, Inc.

4.2.1 Preparation of Insulin Fibrils

+ICD/-ICD insulin fibrils were prepared by our collaborators in Warsaw following published procedures.^(196, 202) Both + and -ICD fibril forms were selected from individual tubes based on the sign and amplitude of their induced ThT CD. Two control samples, were prepared with no agitation, with and without added salt, and are called "Zero-ICD" due to lack of ThT ICD.⁽¹⁹⁶⁾

By contrast, NF and RF insulin fibrils were prepared at UIC following the procedure of Kurouski, *et al.*, using a 70 °C, 24 hours incubation of 60 mg/ml bovine insulin in H₂O/HCl at pH3 and pH1.3, respectively.^(134, 135) The differences in preparation from the +/-ICD sample include no added salt, no agitation, higher temperature and higher concentration in H₂O-based solution. The fibrillation process was terminated by cooling to room temperature and centrifuging the samples at 14,000 x g for 20 min.^(134, 135)

4.2.2 Sample preparations for IR, VCD and TEM

For FTIR and VCD measurements of +/-ICD fibrils, insulin fibrils were suspended in solvent mixtures with variable DMSO/D₂O ratios to acquire a final protein concentration of 0.2 wt% (2 mg/ml). Samples were placed in a sealed homemade cell consisting of two CaF₂ windows separated by a 50 µm Teflon spacer. For FTIR and VCD measurements of NF/RF fibrils, protein concentration was kept constant at 60 mg/ml in variable DMSO/H₂O mixtures, and samples were placed in a BioCell (BioTools, Inc., Jupiter, FL) which has CaF₂ windows with a grooved gap that creates an 8 µm path length. The insulin fibril samples were diluted with DMSO/H₂O solvent to a final concentration of 0.06 mg/ml for TEM measurements. Spectral measurements and TEM sample preparation were done according to the detailed protocols described in Chapter 2.

4.2.3 Induced CD of Insulin Fibril-Bound ThT Measurements

The insulin fibrils were dispersed in ThT solution to a final ThT concentration of 100 μ M and a protein concentration of 0.2 mg/ml. The induced CD spectra of fibrilbound ThT were measured at room temperature using a 1 mm path length quartz cell (detailed protocol for CD measurement was described in Chapter 2). The final spectra were obtained by subtracting the spectrum of a 100 μ M ThT solution (without protein) from the sample spectra.

4.3 Results

4.3.1 DMSO Disassembly of +/-ICD Insulin Fibrils

Insulin fibrils formed by incubating small aliquots of 1 wt% bovine insulin in 100 mM NaCl/D₂O pD 1.9 at 40 $^{\circ}$ C tend to partition into samples that are dominantly either the +ICD or -ICD form as was subsequently determined by the induced CD that develops for fibril-bound ThT.⁽¹⁹⁶⁾ The insulin fibrils can be separated into two different types based on identifying aliquots having oppositely signed induced ThT CD with relatively equal amplitudes. Figure 4.1a shows the induced CD spectra of ThT bound to insulin fibrils for the two forms studied here.

VCD and FTIR spectra of 0.2 wt% of +ICD and -ICD type insulin fibrils are shown in Figure 4.1b. The VCD spectra (Figure 4.1b, top) of +ICD insulin fibril (black line) has an intense positive peak at 1622 cm^{-1} , while -ICD (red line) has a bigger negative peak at 1623 cm^{-1} with two minor positive peaks at 1645 and 1600 cm⁻¹. The VCD sign patterns for + and -ICD are inverted, although the VCD intensity for -ICD is larger than for +ICD by nearly a factor of 2 in this example. In other words, the VCD and ICD intensities are not quantitatively correlated in a single sample. The FTIR spectra (Figure 4.1b, bottom) for both +ICD and -ICD fibrils have the same intensity and amide I' band shapes and are dominated by contributions attributable to a dominant β -sheet conformation indicated by the intense amide I' components at 1627 cm⁻¹ with a weaker side-band at 1660 cm⁻¹, but there is no feature at 1680-1690 cm⁻¹. The +/- ICD insulin fibrils have similar secondary structures based on their identical amide I' FTIR, but the VCD spectra suggest they may differ based on a higher-order, morphological chirality aspect.

+ICD and -ICD insulin fibrils also have opposite signs in their far-UV CD spectra (Figure 4.2); however, these are different from the typical CD bandshape of β -sheet. The +ICD insulin fibril in water has a positive peak around 206 nm, while -ICD has a negative peak around 208 nm with similar intensity as +ICD. The signs of far-UV CD agree with the signs of the VCD and the induced CD of insulin fibril bound ThT in the corresponding samples. These CD sign patterns are consistent with previous reports, although the shapes are a bit different and may be affected by particle scattering effects.⁽¹⁹⁷⁾



Figure 4.1 Formation of chiral variants of insulin fibrils in D₂O. (a) The CD spectra of ThT have been induced by insulin fibrils formed by dissolving 1 wt% of bovine insulin in 100 mM NaCl and D₂O (pD 1.9) at 40 °C for 48 hours. The insulin fibrils were separated into +ICD and -ICD types according to positive (—) and negative (—) CD signs, respectively. (b) The VCD (top) and FTIR (bottom) spectra of 0.2 wt% of +ICD (—, black) and -ICD (—, red) insulin fibrils in 100 mM NaCl and D₂O at pD 1.9.



Figure 4.2 Far-UV CD spectra (in molar ellipticity) of +ICD (black line) and -ICD (red line) insulin fibrils at pH 1.9.
Consistent with previous reports for insulin fibrils, the VCD magnitude for these fibrils is quite high ($\Delta A/A \ge 5 \times 10^{-3}$). Because this enhanced VCD is sensitive to the extent of fibril formation as well as preparation conditions, it was used to monitor the process of DMSO-induced disassembly of insulin fibrils. Mixtures with varying concentrations of DMSO in 100 mM NaCl solution made with pD 1.9 D₂O were added to +ICD and -ICD to obtain final protein concentrations of 0.2 wt% (2 mg/ml) and were left at room temperature for three days to ensure equilibrium. The VCD and FTIR spectra in Figure 4.3a were recorded at room temperature for insulin fibrils with 55 wt% to 90 wt% DMSO. The +ICD and -ICD fibrils (solid lines and dash lines, respectively) behaved similarly at lower DMSO concentrations. The intense VCD is maintained, and the secondary structure appears to be relatively stable even in the presence of 75 wt% of DMSO. With addition of 90 wt% of DMSO, the amide I' band in FTIR spectrum shifted to 1662 cm⁻¹, indicating the β -sheet structure was lost and converted to a DMSO-solvated random-coil-like form.^(199, 203)

However, the VCD spectra of +ICD and -ICD show that they behaved differently over the 75-90 wt% DMSO range. The -ICD fibril evidenced increased intensity for both the positive peak at 1642 cm⁻¹ and negative peak at 1623 cm⁻¹ with 75 wt% DMSO. Under these conditions, the -ICD fibril solution becomes clearer forming soluble β aggregates, as suggested in a previous study.⁽²⁰²⁾ The transparent fibril solution can develop higher VCD intensity than the insoluble fibril possibly due to a reduction of light scattering. Above 75 wt% of DMSO, the -ICD fibril was continuously disaggregated as indicated by the collapse of its VCD intensity and gradual shift of FTIR amide I' band (Figure 4.3b, dash lines).



Figure 4.3 The VCD (top) and FTIR (bottom) spectra were recorded at room temperature for 0.2 wt% +ICD (solid lines) and -ICD (dash lines) insulin fibrils in the mixtures of DMSO and 100 mM NaCl/D₂O (pD 1.9) with varied ratios. (a) The concentration of insulin fibrils was kept constant, while DMSO concentrations in the mixing solvents are 55 wt%, 75 wt%, 80 wt% and 90 wt%. Little change was observed in FTIR and VCD for +/- ICD insulin fibrils with DMSO concentration from 0 wt% - 55 wt%, thus data are not shown. (b) From addition of 80wt% up to 90wt% DMSO, the VCD and FTIR spectra for both +ICD (solid lines) and -ICD (dash lines) started to change. Finally, the VCD intensity collapsed and FTIR amide I' band of both insulin fibrils shifted to higher frequency with 90 wt% DMSO added.

Although the VCD intensity of the +ICD fibril sample decreased at 75 wt% DMSO, it became partially soluble at 80 wt% of DMSO, and showed an increase in VCD intensity (Figure 4.3b, black solid line), as seen for the -ICD at 75 wt% DMSO. However, at 85 wt% of DMSO, conditions under which the -ICD VCD had collapsed and its FTIR significantly broadened, the +ICD sample still evidenced an intense VCD peak and welldefined β -sheet amide I' FTIR (Figure 4.3b, red solid line). Eventually, the collapsed VCD and shifted FTIR amide I' band of +/-ICD fibrils in 90 wt% DMSO solution suggest that they formed similar soluble disaggregated structures, presumably with unordered secondary structures. Somewhat surprisingly, the 90 wt% DMSO samples yielded very small VCD that did not correspond to a measurable PPII-like VCD handshape, which may be due to instrumental artifacts exacerbated by the higher index of refraction for the DMSO solvent.

In order to confirm this result, another set of +/-ICD insulin fibrils was prepared later following the same protocol and mixed with varied concentrations of DMSO in the same manner as described earlier. Although the VCD spectral shape and intensity of this set of +/-ICD insulin fibrils (Figure 4.4a) are different from those shown in Figure 4.1b due to sample variation, FTIR and the inverted VCD sign patterns of +/-ICD are reproducible. As shown in Figure 4.4a, +ICD has a negative-positive-negative VCD sign pattern with a deeper negative peak at higher frequency and the intensity is larger than -ICD by almost a factor of 2, which is opposite of the Figure 4.1 intensity ratio.



Figure 4.4 Another set of +/-ICD insulin fibrils was prepared later using the same protocol. (a) VCD (top) and FTIR (bottom) spectra of 0.2 wt% +ICD (black line) and -ICD (red line). (b) The VCD and FTIR spectra of +ICD and -ICD insulin fibrils in the mixtures of DMSO and 100 mM NaCl/D₂O (pD 1.9) with varied ratios (70 wt%, 85 wt% and 90 wt%).

Similarly to the previous samples, the new +ICD and -ICD insulin fibrils were stable at lower concentrations of DMSO, and -ICD was again destabilized prior to +ICD at high concentration of DMSO resulting in a collapsed VCD and gradually shifted amide I' FTIR, while +ICD still maintained a relatively high VCD intensity and an evident β -sheet component in its FTIR spectrum, even at 90 wt% DMSO (Figure 4.4b). However, as opposed to the set of samples whose data is in Figure 4.3, +ICD and -ICD in the alternate set of samples were not fully denatured at 90 wt% of DMSO even after long incubation times. This was evidenced by broadened amide I' FTIR with multiple components including significant intensities at lower frequency. These results indicate the degree of disaggregation at 90 wt% DMSO may be fibril preparation dependent. Overall, although the VCD intensities did change with sample variation, the qualitative trend was reproducible. The +ICD and -ICD fibril forms have a different resistance to DMSO denaturation as evidenced by their VCD intensity change and FTIR amide I' band shift during these structural transitions.

Our collaborator did AFM images on +/-ICD insulin fibrils that were suspended in concentrated DMSO (70-90 wt%). They observed that +/-ICD at 70-80 wt% DMSO still formed large aggregates clustering together on the mica surface used for AFM. However, at 90 wt% of DMSO, the size of larger aggregates was significantly decreased resulting in a distribution of small particles, that implies a disassembly of +/-ICD insulin fibrils at high concentration of DMSO, which is consistent with the VCD and FTIR spectral results.

Insulin fibrils prepared without agitation have no ICD for fibril-bound ThT, and are termed "Zero-ICD".⁽¹⁹⁶⁾ In comparison with +/-ICD, "Zero-ICD" insulin fibrils have

weaker VCD and broadened amide I' FTIR (Figure 4.6). VCD spectra for "Zero-ICD" formed with no salt added were orientation dependent, presumably evidencing some self-assembly, and its intense β -sheet amide I' FTIR was quite broad with two main components (Figure 4.6a). This orientation dependence could be eliminated by sonication of the sample, but then only very weak VCD was detectable. The second "Zero-ICD" sample, a heterogeneous aggregate formed with added salt, had very weak VCD with low signal-to-noise possibly due to the larger clusters formed. After brief sonication, a more uniform suspension resulted had a negative-positive (high to low) VCD couplet peaking at 1637 and 1612 cm⁻¹, respectively (Figure 4.6b). Because of their complex VCD behaviors, no DMSO disassembly studies were performed for either of these "Zero-ICD" insulin fibrils. The TEM image of "Zero-ICD" formed with no salt shows networks of long, thin fibrils (Figure 4.5a) has a different morphology (gel), tending toward shorter and thicker bundles (Figure 4.5b).



Figure 4.5: TEM images of (a) "Zero-ICD" formed with no salt and (b) "Zero-ICD" formed with salt, the scales are 200 nm.

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b



Figure 4.6 VCD and FTIR spectra of 1 wt% "Zero-ICD" insulin fibrils. (a) The first "Zero-ICD" insulin fibril was formed with no salt addition and no agitation has broadened amide I' FTIR and oriented VCD. The orientated dependence of the VCD, determined by measurement after rotating it every 90°, can be eliminated by brief sonication of sample, but the VCD intensity will significantly drop resulting in a very low signal-to-noise. (b) The second "zero-ICD" formed in salt solution without agitation shows very weak VCD and broadened amide I' FTIR (black line). After brief sonication, this sample becomes homogenous and shows "negative-positive" coupled VCD peak with narrowed amide I' FTIR (red line).

4.3.2 DMSO Disassembly of NF an RF Fibrils

In order to investigate the effect of DMSO on different forms of insulin fibrils, NF and RF insulin fibrils formed in H₂O/HCl with no agitation or salt were similarly studied to compare with the above results from +ICD and -ICD insulin fibrils. The higher temperature, concentration and variable pH used for the NF and RF preparations appear to result in a morphological form distinct from that of the "Zero-ICD" one noted above, despite otherwise similar preparations. The insulin fibrils formed at pH 3 (Figure 4.7a, NF red line) show an intense positive-negative-positive VCD sign pattern as have been extensively reported by Lednev, Nafie and co-workers.⁽¹³⁴⁾ By contrast, RF fibrils formed at pH 1.3 (Figure 4.7a, RF black line) have generally the opposite VCD sign pattern, again as previously reported.^(134, 135) For our preparations, the RF fibrils give rise to much weaker VCD than do the NF (Figure 4.7b), and even the NF VCD are somewhat weaker in terms of $\Delta A/A$ than that we obtained for +/-ICD insulin fibrils. The VCD intensities are variable for those particle suspension samples. Furthermore, they will be instrument sensitive due to baseline variations and spectral resolution.

Far-UV CD spectra of NF and RF insulin fibrils are similar (Figure 4.8), both showing bandshapes with a negative peak around 216 nm, which is consistent with the CD pattern expected for the β -sheet core secondary structure as determined by FTIR (Figure 4.7c).

The NF and RF insulin fibrils were dispersed in DMSO/H₂O solvent mixtures in the same manner as above for \pm -ICD but at much higher concentration (60 mg/ml), which is needed along with the shorter path length for these H₂O based solution studies.



Figure 4.7 The VCD (a,b) and FTIR (c) spectra of NF and RF insulin fibrils. (a) The insulin fibrils formed at pH3 water (NF —, red line) and at pH 1.3 (RF —, black line) had flipped VCD signal pattern. For better comparison, the VCD intensity of RF was multiplied by factor of 5. (It might be noted that we were not successful in attempts to prepare NF/RF fibrils in D₂O based solvents with the correct handshape.) (b) The actual VCD and (c) FTIR spectra of NF and RF fibril forms. The VCD intensity of RF is less than NF. Both of them showed FTIR amide I band at 1630 cm⁻¹ with a minor peak at 1660 cm⁻¹.



Figure 4.8 Far-UV CD spectra (in molar ellipticity) of NF (black line) and RF (red line) insulin fibrils at pH 3 and pH 1.3, respectively.

Figure 4.9 shows the changes in VCD and FTIR spectra for the NF form when the DMSO fraction varies from 50 - 90 wt%. The FTIR spectra for NF at 50-70 wt% DMSO were qualitatively consistent, representing a fairly stable dominant β -sheet secondary structure. However, although the VCD spectrum of NF fibril in 50 wt% DMSO (black solid line) was about the same as for low concentration DMSO (Figure 4.9), at 60 wt% DMSO the VCD peak sign flipped (red dash line) and then decreased in intensity for 70 wt% DMSO (blue dot line). The FTIR for NF in 80 and 90 wt% DMSO indicates a conversion to a DMSO-solvated unordered structure, and the associated VCD collapsed (Figure 4.9 and Figure 4.10). By contrast, the VCD spectra of RF fibril in 50 - 90 wt% of DMSO remain very weak but have a roughly stable form up to 60 wt%, then start to decrease at 70 wt% while maintaining a very weak negative peak around 1645 cm⁻¹ and no change in sign pattern, but become virtually undetectable at 80 and 90 wt% (Figure 4.11, baseline degradation by DMSO). The amide I band FTIR broadens with DMSO addition but still indicated a minor β -sheet component even at 90 wt%. These trends are consistent but the VCD intensity and degree of IR broadening at higher DMSO concentrations varies with samples isolated.

Since the VCD chirality may be related to the macromolecular morphology, TEM was used to visualize the fibril morphology under various sample conditions. The TEM images for NF fibrils in H₂O pH 3 showed more aggregated and twisted fibrils (Figure 4.12, top), which are different from and thinner than the RF fibrils which have thicker, less aggregated forms, more like flat ribbon-shapes (Figure 4.12, bottom), as has been previously reported.^(134, 135) The NF fibril in 50 wt% DMSO still maintained a twisted morphology, but was more disaggregated (Figure 4.13, top).



Figure 4.9 The VCD and FTIR spectra of 60 mg/ml insulin fibrils in the DMSO/H₂O mixture. With addition of 50 - 90 wt% DMSO/H₂O into NF insulin fibrils at pH 3, the VCD signal flipped then collapsed.



Figure 4.10 The Amide I range of NF insulin fibril in 80% and 90% $DMSO/H_2O$ mixtures. The FTIR Amide I band shifted to higher frequency and VCD spectra collapsed presumably due to unordered structure. Since DMSO has a big influence on VCD baseline, only the spectra of amide I range were shown.



Figure 4.11 The VCD and FTIR spectra of RF insulin fibril in 50% - 90% (top to bottom) DMSO/H₂O. The general broadening for FTIR corresponds to a weakening of the VCD, but the same sign pattern persists to 60 wt%, and collapses at 70 wt%. Adding DMSO degrades baseline flatness in this VCD instrument, and extra noise reflects lack of clarity in the sample.



Figure 4.12 The TEM images of NF fibril (top) and RF fibril (bottom), the scales are 200 nm.





Figure 4.13 The TEM images of NF fibril in 50 wt% DMSO/H₂O mixture (top) and NF fibril in 70 wt% DMSO/H₂O mixture (bottom). The arrows indicate of multistrand twist in the NF fibril at 50 wt% DMSO. The scales are 200 nm.

However, at 70 wt% DMSO, it transformed to flat ribbon shaped fibrils (Figure 4.13, bottom), which are similar to RF fibrils as have been previously described using AFM and SEM data.⁽¹³⁴⁾ Consistent with our VCD spectral results, the TEM morphology indicates that, with higher concentration of DMSO added, an inter-conversion occurred from NF to RF, or from twisted to ribbon.

In order to investigate the cause of the flipped VCD sign of NF fibril in DMSO, additional tests have been carried out. DMSO was removed from 60 wt% and 70 wt% NF insulin fibril samples by centrifugation and discarding the supernatant. Afterwards, the aggregated samples were resuspended into H₂O with pH adjusted to 3 and then the suspension was divided into two parts. One part of the sample was incubated at room temperature and another was heated at 70 °C. After 3 days incubation at room temperature, the VCD spectra of both resuspended 60 wt% and 70 wt% NF stayed flipped (Figure 4.14). By contrast, after thermal incubation at 70 °C for 24 hours, the resuspended "60 wt% NF" was partially converted back to its original NF VCD sign pattern but with less intensity (Figure 4.15). However, this conversion was not stable, such that after re-incubating the same sample at room temperature for one day, the VCD spectra returned to the flipped sign (Figure 4.15).



Figure 4.14 VCD (top) and FTIR (bottom) spectra of NF conversion test in 60 and 70 wt% DMSO. (a) The NF fibril with 60 wt% DMSO sample was resuspended into H₂O (pH3) after the removal of DMSO by centrifugation. The sample has been incubated at room temperature for 3 days. (b) The NF fibril with 70 wt% DMSO (black dash line) was treated in the same way as the 60 wt% sample, NF fibril was resuspended into H₂O (pH3) and incubated at room temperature (blue line) and at 70 $^{\circ}$ C (red line). None of these conditions can convert the VCD spectra back to normal NF type.



Figure 4.15 VCD (top) and FTIR (bottom) spectra of NF conversion test in DMSO. After removing the DMSO from 60 wt% DMSO NF fibril, the fibril was resuspended into H₂O (pH3) and incubated at 70 $^{\circ}$ C (fibrillation temperature) for 24 hours. It was partially converted to normal NF VCD (black solid line), but after incubating this same heated sample at room temperature for one day, its VCD spectra converted back to flipped sign (red dash line).

4.4 Discussion

4.4.1 DMSO-Induced Disassembly of +/-ICD Insulin fibrils is confirmed by VCD

Amyloid fibrils have common overall secondary structural properties, but their morphologies can vary based on fibrillation conditions (temperature, pH and ionic strength), the assembly of protofilaments and precursor protein.^(88, 204) DMSO-induced destabilization of insulin fibrils to form small soluble oligomers with an amyloid-like secondary structure conformation was previously studied by FTIR and AFM.⁽²⁰²⁾ In this study, we were able to compare two types of insulin fibrils prepared under different conditions that resulted in different aggregation states and different spectral properties regarding to their interaction with and disassembly by added DMSO as monitored by VCD and FTIR. It needs to be noted that these two types of insulin fibrils have distinct far-UV CD spectra in the amide n- π^* and π - π^* region normally used to determine the dominant secondary structure. +ICD and -ICD have oppositely signed but differently shaped CD spectra between ~225-200 nm (Figure 4.2), $^{(197)}$ while both NF and RF have a weak negative peak at 216 nm which is typical for a β -sheet structure Figure 4.8). Thus while VCD discriminates between morphological chirality, the far-UV is much less clear, especially for NF/RF insulin fibrils, CD only reflects secondary structure but not morphology.

The +/-ICD form was prepared in 100 mM NaCl/D₂O at pD 1.9 with high speed agitation. Based on the induced CD of fibril-bound ThT, the resultant samples can be separated into +ICD and -ICD types as shown in Figure 4.1a. The VCD spectra could similarly discriminate between these types, showing that the fibril VCD peak patterns have a variation consistent with that of the ICD results (Figure 4.1b). This indicates that

both ICD and VCD methods sense the fibril chirality and do not report on the local amide secondary structure for these aggregates. Thus, +ICD and -ICD insulin fibrils are different in terms of morphological chirality, although they have same secondary structure as evidenced by their similar FTIR amide I' band shapes (Figure 4.1b). The distinct VCD spectra for the two ICD forms appear to correspond to chirality differences in the higher-order fibril structures.⁽¹⁹¹⁾

It can be noted that the FTIR amide I' maximum for the +/-ICD samples occurs at a higher frequency than is typical for a well-ordered extended antiparallel β -sheet. Amide I' bands for model antiparallel sheets might be expected to be at ~1610-1620 cm⁻¹ with a low-intensity peak at ~1680-1690 cm⁻¹.^(153, 205-208) One possible explanation is that there are several secondary structural components in the insulin fibril, and the observed shifted amide I' maximum and broadened shoulder may be due to residual structures likely including loops and turns as has been postulated from peak fitting results.⁽²⁰⁹⁾ Since the FTIR spectra lack a high frequency component (~1685 - 1690 cm⁻¹) characteristic of antiparallel β -sheet structure,^(210, 211) it is possible that the β -sheet component in insulin fibrils has a significant parallel rather than antiparallel conformation based on amide I IR bandshapes determined for model β -sheets in previous experimental and theoretical studies.^(88, 153, 204, 208) However, in order to conclusively determine the structural arrangement of the insulin fibril β -sheet assemblies, further studies are needed, such as isotope labelling, as commonly used for model peptides,^(153, 212-214) or protein mutations.⁽²¹⁵⁾

The +/-ICD fibrils retained β -sheet-like FTIR amide I' spectra and evidenced enhanced VCD up to the addition of 75 wt% of DMSO (Figure 4.3a). At high DMSO

concentrations, the -ICD was destabilized prior to +ICD, but both + and -ICD fibrils were converted to a partially or fully DMSO-solvated disordered structure with the same VCD and FTIR spectral patterns at 90 wt% DMSO. The final VCD and IR spectral band shapes and degree of denaturation of the fibril at 90 wt% DMSO are sample dependent as determined from comparison of different sets of +/-ICD samples.

4.4.2 NF/RF Insulin Fibrils showed Different VCD Behaviors to Added DMSO

The alternate NF and RF forms of insulin fibril studied were obtained by thermal incubation of bovine insulin in H₂O/HCl at two different pH values that result in oppositely signed VCD.^(134, 135) As noted earlier, the spectra of NF and RF fibrils in D₂O are sensitive to sample concentration and dilution, thus DMSO-induced disassembly of NF and RF fibrils was studied in H_2O , which is additionally consistent with published H₂O-based data. The NF and RF types have been related to distinctly different, AFMdetermined supramolecular morphologies, as described earlier by Kurouski et al.^(134, 135) Consistent with their studies, our TEM results indicate that the NF insulin fibril has a long, thin and twisted morphology (Figure 4.12, top) that results in an enhanced VCD signal, while the RF insulin fibril appeared to have a short and wide flat ribbon morphology (Figure 4.12, bottom) and evidenced weaker VCD (Figure 4.7b). However, neither the NF nor the RF fibril forms that we prepared showed significant ThT ICD above our baseline (\sim -1 mdeg), which is less intense than data reported by others.⁽¹³⁴⁾ Although NF/RF and "Zero-ICD" insulin fibrils both have little or no ThT ICD, their VCD spectra are quite different due to the different sample preparation conditions used to obtain them.

However, in contrast to the +/-ICD fibrils, the VCD sign of the NF fibril flipped and remained relatively intense in 60 and 70 wt% DMSO solutions, although the amide I FTIR for NF in these solutions maintained a consistent shape characteristic of β -sheet secondary structure (Figure 4.9). These spectra indicate that the NF fibril was first converted to an "RF" type in 60 and 70 wt% DMSO solutions without changing its underlying β -sheet structure as demonstrated by the VCD sign reversal with no accompanying FTIR change (Figure 4.9). This is supported by the corresponding change in the TEM images (Figure 4.13). The NF fibrils were eventually disordered in 80 and 90 wt% DMSO (Figure 4.10).

Although the initially prepared RF fibril with DMSO had much weaker VCD with low signal-to-noise, relatively stable β -sheet dominant secondary structure is still evident from FTIR spectra for the RF fibrils in DMSO even with 80 wt% added (Figure 4.11). RF still retained minor β -sheet components at 90 wt% DMSO, and thus appears to be more resistant to DMSO disaggregation than is the NF fibril. Comparing the +/-ICD and NF/RF insulin fibril types as to resistance to DMSO disassembly, +/-ICD have relatively higher resistance based on relatively stable VCD and FTIR spectra at high DMSO concentration. For the +/-ICD insulin fibrils, although the presence of salt can promote the fibrillation process to form superstructure through electrostatic interaction, ^(216, 217) the high ionic strength may counteract the DMSO-induced destabilization of the insulin fibril by limiting accessibility of DMSO to the interior of the multi-stranded fibril. Possibly more important, the higher degree of strand aggregation and braiding in the +/-ICD (Figure 4.3) as compared to NF and RF types (Figure 4.12) may impede DMSO solvation of individual fibrils to eventually break up their cross-beta structures

4.4.3 An Irreversible Conversion from NF to RF Occurred During DMSO-Induced Destabilization Confirmed by VCD and TEM

Since the VCD can provide information related to fibril morphology and potentially its macrochirality,⁽¹³⁴⁾ we propose that the flipped VCD sign of NF fibril in 60 and 70 wt% DMSO may be caused by inter-conversion of fibril forms. NF fibrils are observed to be twisted and long with 50 wt% DMSO, but our TEM images suggest that these change to short, ribbon-like structures at 60 and 70 wt% DMSO, which is consistent with the flipped VCD sign. The removal of DMSO solvent from 60 and 70 wt% of NF did not manage to convert the flipped VCD sign pattern back to normal NF VCD sign at room temperature (Figure 4.14). Although the "60 wt% NF" can be temporally partially converted back to normal NF VCD with less intensity by thermal incubation, it failed to maintain the normal NF VCD at room temperature (Figure 4.15). It suggests an irreversible intermediate formed during the DMSO-induced destabilization process.

Thus, in summary, with addition of 60 wt% of DMSO, NF type was converted to RF type and formed a relatively stable structure, which is not easily changed back just by elimination of DMSO and substitution of H₂O at pH3. At 60 wt% of DMSO, the NF insulin fibril appears to start inter-conversion with changes in morphology and flipped VCD sign patterns. This structure is stable at room temperature, but less stable at high temperature. In contrast, at 70 wt% of DMSO, NF type was fully converted to a stable RF type with morphology changed from twisted fibril to flat ribbon, and it has high stability even at elevated temperatures. An inter-conversion of NF and RF insulin fibrils was previously reported by Kurouski *et al*, who showed that RF fibril can be converted to NF fibril by elevating the pH value during the fibrillation process.⁽¹³⁴⁾ However the change

induced by DMSO addition is the opposite direction, from NF to RF, and seems to be irreversible. Since this conversion was observed in both fibril assembly and disassociation processes, it may suggest that the RF type of insulin fibril is an intermediate formed first at low pH during the fibrillation process, which can be relatively incomplete (shorter, thinner structure) and provide the basic building blocks in NF fibril formation at pH 3. In the reversal process on adding DMSO to fully developed, more aggregated NF fibrils, these twisted filaments were gradually dissociated and finally formed the "RF-like" intermediate which is now longer and more complete and can pose a kinetic trap, making reformation of NF impossible, even if DMSO is removed. Such an apparent reversal of relative stabilities is difficult to explain other than by a kinetic trap, suggesting that insulin may provide an interesting model system for exploring the rugged landscape for folding and misfolding.

4.5 Conclusion

Although the insulin fibrils have similar secondary structures, their high-order chirality and supramolecular morphologies vary based on fibrillation conditions. The VCD sign pattern is sensitive to highly ordered superstructures, thus it can be used to distinguish various types of amyloid fibril morphologies. The VCD of DMSO-induced insulin fibril destabilization showed that insulin fibrils were relatively stable at low concentrations of DMSO and were eventually disaggregated into DMSO-solvated disordered structures at very high DMSO concentrations. However, the behaviors at relatively high, but less than maximal DMSO concentrations were sample dependent. The + and -ICD insulin fibrils were prepared with high salt concentration and high speed agitation and have relatively high resistance to DMSO, possibly due to the lower access

of DMSO to the proteins in the highly aggregated fibrils. The conversion from NF type to RF type at relatively high concentration of DMSO suggests the twisted NF fibril is dissociated and untwisted to a RF-like irreversible intermediate (kinetic trap) in the disassembly process. This intermediate in a less developed form may also occur prior to the formation of mature NF fibril in the fibrillation process.

5. Refolding and Characterization of A Computationally Designed Outer

Membrane Protein

This chapter is mainly focused on the experimental part of an ongoing research project, which is a collaboration with Ms. Meishan Lin from Prof. Jie Liang's group at UIC, who developed the computational design for the protein.

5.1 Overview

Outer membrane proteins (OMPs) existing in gram-negative bacteria can form channels in the membrane for transporting various solutes and filtering out unwanted molecules.⁽⁹⁵⁾ These OMP channels are classified into three types: classical porins (general porins), substrate-specific channels and active transporters.⁽⁹⁵⁾ The classical porins, such as OmpF and OmpC, generate pores allowing small polar solutes (< 600 Da) to travel through the membrane without any preference and being driven by a concentration gradient across the outer membrane.⁽⁹⁰⁾ The substrate-specific channels, such as LamB and Tsx,^(218, 219) are very useful for transporting some low concentration substances which can specifically bind with channels. The active transporters, like FhuA and FepA, can bind specific substrates with high binding affinity and transport them against diffusion.^(220, 221)

The non-specific general porins have been widely studied due to their well-defined β -barrel structure and important biological functions.^(89, 90) As described in Chapter 1, one well-studied porin in *Escherichia coli*, OmpF, is stabilized in the membrane as trimeric 16-stranded β -barrels containing constriction regions in the center of the barrels (Figure 1.13).⁽⁹⁰⁾ Its channel conductivity is to some degree confined by the restriction regions inside the barrel. In contrast to general porins, OmpG in *Escherichia coli* K-12 is a

special one. OmpG stabilizes as 14-stranded β -barrel monomer and forms a relatively highly permeable channel (Figure 1.14), due to lack of monomer-monomer interface or a restricted loop region.⁽⁹³⁾ However, the channel conductivity of OmpG is also affected by its pH-mediated gating behavior, and it adopts open and closed conformations at neutral and acidic pH, respectively.^(92, 222) Additionally, it has been widely reported that OmpF and OmpG in a urea-denatured state can fold in lipid bilayer or in detergents under proper buffer conditions (ionic strength, pH and reduction in urea concentration).^(96, 223-225)

In order to optimize the channel conductivity and selectivity for transporting small drug molecules or for developing new biosensors, many research studies have focused on synthesizing and engineering multifunctional pores on the basis of existing OMPs. One common approach is to mutate key amino acid residues. For example, multiple mutations of general porins to eliminate the L3 loop and widen the pore can significantly increase the permeability of channels.⁽²²⁶⁾ Mutagenesis on OmpG to reduce the mobility of the L6 loop, which is attributed to OmpG gating activity, can effectively stabilize the open conformation of OmpG based on molecular dynamics simulation studies.⁽²²⁷⁾ Alternatively, engineered pores can also be obtained by duplicating or deleting multiple amino acid sequences, for example, the channel diameter of FhuA increases by doubling the amino acid sequences of first two β -strands at N-terminus;⁽²²⁸⁾ and the deletion of 8 or 12 amino acid residues on the L6 loop results the formation of a minimal OmpG with loop independent gating behavior.⁽²²⁹⁾

Based on computational sequence motif and empirical energy function studies,^(230, 231) our collaborators found that the first six β -strands of OmpF are less stable, and these strands compose the monomer-monomer interface (L2 loop) and restriction region in the

barrel (L3 loop). Similarly, the unstable region in OmpG is from β -strand 6 to 11, including the L6 loop, which is highly involved in the gating activity. Based on the prediction of stability and protein-protein interaction, they designed a new 16-stranded β -barrel porin based on OmpF (16-stranded β -barrel) but substituting the unstable β -strands 1-6 of OmpF with stable β -strands 1-6 from OmpG, to create a new porin, so called OmpFG. Since the constriction region and monomer-monomer interface of OmpF have been removed, OmpFG is proposed to form a monomer β -barrel with high channel conductivity.

In this study, we focus on the refolding and characterization of OmpFG, in comparison to parallel studies of OmpF and OmpG, using multiple techniques including CD, fluorescence, SDS-PAGE and a dye leakage assay. It should be noted that most OMPs show a common characteristic, called "heat modifiability". For SDS-PAGE gel separation studies, SDS molecules in the loading buffer can trap folded OMP fractions in solution.^(232, 233) The captured folded fractions are denatured by heating the sample at 95 \mathbb{C} for 5 min, so that they lose their native β -barrel structure and are observed at the expected position on the SDS-PAGE gel based on their molar weight.⁽²³³⁾ The unheated samples still maintain any folded conformation and migrate to different positions from the heat-treated ones.⁽²²⁴⁾ Thus heat modifiability in SDS-PAGE has been extensively used for discrimination between OMP folded and unfolded fractions.

5.2 Materials and Methods

Lipids, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Inc. DLPC has two saturated 12-hydrocarbon chains with phase transition of -2 °C. DOPC has two

unsaturated 18-hydrocarbon chains with phase transition of -17 °C. Detergents, ndodecyl- β -D-glucopyranoside (DG), n-dodecyl- β -D-maltoside (DM) and n-octyl- β -Dglucopyranoside (OG) were purchased from Sigma. For CD and fluorescence measurements, the molar concentration of OMPs used was 4 μ M (0.15 mg/ml OmpF, 0.13 mg/ml OmpG and 0.14 mg/ml OmpFG). The procedures for lipid vesicle preparation (SUV and LUV), CD and fluorescence measurements were described in detail in Chapter 2.

5.2.1 Cloning and Expression of OMPs

The synthetic gene "OmpFG" was subcloned by GenScript (Piscataway, NJ) into an *E. coli* expression vector pET11a using NdeI and BamHI sites to construct a plasmid. OmpF and OmpG genes were constructed into pET28a (+) vectors with BamHI and XhoI sites in Prof. Liang's lab.⁽²³⁴⁾ The resultant OMP plasmids were transformed into BL21 (DE3) StarTM cells (Invitrogen).

The transformed cells were grown at 37 °C in 200 ml LB medium (LB Broth, Fisher Scientific) containing 100 µg/ml ampicillin for OmpFG and 50 µg/ml kanamycin for OmpF and OmpG. The cultures were induced by 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when the optical density reaches around 0.6. After 3 hours induction, the cells were harvested by centrifugation (8000 rpm, 20 min, 4 °C). The cell pellets were resuspended in 8 ml 1xTE buffer (tris, EDTA and MgCl₂, pH7) containing 10 µl 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 50 µl of 20 mg/ml lysozyme. The lysed cells were incubated on ice for 40 min and then sonicated for 7 cycles with 30 seconds for each cycle. The inclusion bodies were collected by centrifugation (7000 rpm, 30 min, 4 °C) and washed three times using 12 ml TE buffer

containing 1 % triton. After the washing steps, the washed inclusion body pellets were recollected by centrifugation and stored at -20 °C.

5.2.2 Protein Purification

The OMP inclusion bodies were dissolved in a denaturation buffer (50 mM Tris-HCl, 8 M urea, pH 8.0) and incubated at room temperature for 30 min. The undissolved cell pellets were removed by centrifugation (13000 rpm, 15 min), and the supernatant was passed through a 0.22 µm filter before purification. The urea-denatured OMPs were eluted with 0 to 0.5 M NaCl gradient in the denaturation buffer using an ion exchange column packed with Q Sepharose Fast Flow strong anion resins (GE Healthcare Life Sciences, Pittsburgh, PA).⁽²²⁵⁾ After collecting all the elution fractions, the purified fractions were determined by SDS-PAGE analysis. OmpF, OmpG and OmpFG were eluted at NaCl concentrations of 75 mM, 200 mM and 160 mM, respectively. The concentrations of purified proteins were determined by absorbance at 280 nm using a UV-vis spectrometer, as described in Chapter 2. The extinction coefficients of OmpF (48500 M⁻¹cm⁻¹), OmpG (84950 M⁻¹cm⁻¹) and OmpFG (66140 M⁻¹cm⁻¹) were calculated using Protein Extinction Coefficient Calculator (Bioml.net).

5.2.3 Refolding of OMPs using Lipid Vesicles or Detergents

The urea-denatured OMPs were refolded by diluting into 8 mM Lipid SUVs or LUVs in refolding buffer (1 M urea, 2 mM EDTA, 20 mM borate, pH 9.6) to a final concentration of 4 μ M OMPs and 5 mM lipid vesicles. The refolding process was completed by incubation at 30 °C for 19 hours with 60 rpm gentle shaking.⁽²²⁴⁾

OMPs can also be refolded by mixing with detergents. The urea-denatured OmpF was diluted into detergent refolding buffer (0.5 % DG and 0.2 % DM in 50 mM Tris-HCl,

ImM DTT (dithiothreitol) and 0.1 mM EDTA, pH 8) by 10-fold to the final OmpF concentration of 4 μ M.^(234, 235) The mixture of OmpF and detergent was incubated at 37 \mathbb{C} overnight. The refolded OmpF trimer and dimer (> 50 kD) were concentrated using Amicon Ultra-0.5 ml Centrifugal Filters 50K (Millipore). The urea-denatured OmpG was refolded by diluting into OG refolding solution (20 mM Tris-HCl, 3.33 % OG, pH 9.0) to a final concentration of 12.8 μ M OmpG, 2 % OG and 3 M urea. The mixture was incubated at 37 \mathbb{C} over 24 hours to reach a maximum folding efficiency.⁽²²⁵⁾ The urea-denatured OmpFG was diluted into the OmpF detergent refolding buffer and the OmpG detergent refolding buffer in the same manner as described above.

5.2.4 SDS-PAGE

The refolding process of OMPs was quenched by adding 5x SDS loading buffer. The refolded OMPs samples were trapped by SDS and split into two parts. One part was directly loaded onto 4-20 % acrylamide gradient gel (Bio-Rad) without heating to maintain the folding conformation. The other part was denatured before loading by heating at 95 °C for 5 min using a thermal bath (Labnet, Inc.). Each sample is loaded with 15 μ l. After electrophoresis, the gels were stained with Coomassie Blue using gentle shaking overnight and destained for imaging the gel.

5.2.5 Dye Leakage Experiment

The dried DLPC lipid film was hydrated by 120 mM 5(6)-carboxyfluorescein (Sigma) in refolding buffer (1 M urea, 2 mM EDTA and 20 mM borate pH 9.6). The suspended lipid solution was sonicated to form 12 mM DLPC SUV. The lipid vesicles capturing carboxyfluorescein inside were separated from the solution containing the remaining untrapped dye using a Sephadex G-50 size exclusion column. The vesicles with most

concentrated quenched dyes trapped inside were selected based on most reduced fluorescence intensity with excitation wavelength at 490 nm and emission wavelength at 520nm. The urea-denatured OMPs were added into the selected lipid vesicles and the mixtures were incubated at 30 \mathbb{C} for 19 hours with 60 rpm shaking. Before and after the refolding process, the fluorescence spectra were recorded for lipid vesicles with and without refolded OMPs. The maximum leakage was determined by adding 10% triton to the lipid vesicle solutions, resulting in the complete disruption of the vesicles.^(236, 237) The leakage percentage is calculated by following equation:

Leakage percentage =
$$\frac{(F - F_0)}{(F_{100} - F_0)} \times 100\%$$
 (5.1)

F: fluorescence intensity of OMPs with lipid vesicles after incubation F_0 : fluorescence intensity of DLPC SUV without OMPs after incubation F_{100} : fluorescence intensity of DLPC SUV with addition of 10 % triton, which results in complete disruption of vesicles.

5.3 Results

5.3.1 Refolding of unpurified OMPs using Lipid Vesicles

In this study, two lipids with PC head groups (DLPC and DOPC) but different length and property of hydrocarbon chains were used to refold OMPs following literature protocols.⁽²²⁴⁾ The urea-denatured OMPs were mixed with DLPC and DOPC SUVs using an incubation period of 19 hours at 30 °C to reach optimized folding efficiency. Shown in Figure 5.1 is a comparison based on their variation in mobility due to heat denaturation, OmpF was folded in DLPC SUVs to form trimers (T) and dimers (D) which appear at distinct positions in the SDS-PAGE gel with different folding efficiencies (more dimer

band intensity than trimer). It may also form a monomer fraction that could appear at a position very close to that of the unfolded fractions, thus the monomer cannot be identified, which is consistent with the literature reports.⁽²²⁴⁾ No folded fraction was observed for OmpF with DOPC SUVs. OmpG with DLPC SUVs formed a monomer at lower position in the gel than unfolded fractions by comparing heated and unheated samples. However, OmpG with DOPC SUV showed only a barely visible folded band indicating the folding efficiency of OmpG in DOPC bilayer is much lower than in DLPC. However, for OmpFG samples, no separate folded bands can be observed with either DLPC or DOPC SUV.

In order to investigate the effect of vesicle curvature on refolding of OMPs, DLPC SUVs and LUVs were used to compare the folding efficiency. OMPs were refolded with DLPC SUVs and LUVs following the same procedure, based on the SDS-PAGE gel analysis, OmpF and OmpG can be refolded in both SUV and LUV solutions (Figure 5.2). However, the folding efficiency of OMPs with SUVs and LUVs is slightly different. OmpF formed more dimer and less trimer fractions in DLPC SUV solution with higher overall folding efficiency of OmpG in DLPC SUVs and LUVs. Consistently, no separated folded fraction was observed in OmpFG samples. However, OmpFG monomer may have behavior like the OmpF monomer, which has no heat modification of mobility evident in the gel. To confirm this hypothesis, alternate methods need to be employed to check the refolding of OmpFG.



Figure 5.1 Heat modifiability of OMPs determined by 4-20 % gradient SDS-PAGE. The urea-denatured OMPs were refolded into DLPC and DOPC SUVs in refolding buffer (1M urea, 2 mM EDTA, 20 mM borate, pH 9.6) After 19 hours and 30 $^{\circ}$ C incubation, refolded samples were captured by adding 5x SDS-PAGE loading buffer. The heated and unheated samples were labelled with "+" and "-" on top, respectively Folded (F) and unfolded (U) fractions were labeled on the right side of corresponding bands. For OmpF, the folded trimer (T) and dimer (D) fractions were also labeled. Unfolded OMPs in denaturation buffer (8 M urea) are shown in the lane 2, 6, and 11.


Figure 5.2 The refolding of OMPs in DLPC SUV and LUV determined by 4-20% gradient SDS-PAGE. The unheated and heated OMP samples were labelled with "+" and "-" on top, respectively. Folded (F) and unfolded (U) fractions were labeled on the right side. The folded OmpF trimer (T) and dimer (D) were also labeled. The unfolded OMPs samples in the denaturation buffer (8 M urea) are in the lane 2, 7 and 12.

If OMPs fold into β -barrel structures in the lipid bilayers, they should evidence stable secondary structure containing a large fraction of β -sheets which can be monitored by circular dichroism or other spectral methods. Unfolded OMPs without purification were directly diluted into DLPC SUV in refolding buffer (1 M urea, 2 mM EDTA and 20 mM borate, pH 9.6). It was reported that refolding buffer containing 1 M urea provides a suitable condition for OMPs to be partially folded.⁽²²⁴⁾ As shown in Figure 5.3, in the 8 M urea denaturation buffer, unfolded OMPs showed little CD above 210 nm, which is consistent with the protein having a random coil structure (blue lines, —). However, in 1 M urea with DLPC SUVs, all three OMPs showed CD consistent with partial formation of β -sheet structures with negative extrema close to ~ 216 nm (black lines, —), especially for OmpF and OmpFG. In the refolding buffer (1 M urea) without lipid vesicles, OMPs adopted some degree of folded structure with enhanced intensity and broadened bands around 215-210 nm. The low signal-to-noise of the spectra may be due to the background effect of urea, low concentrations used and OMP impurities.

In order to further distinguish the folded (with lipid) and partially folded (just in refolding buffer) fractions using CD spectra, a cuvette with shorter path length of 0.5 mm (half of the previous path length) was used for CD measurement. Although a shorter path length will lower the CD signal level, it can enable reliable spectra to be measured further into the far-UV range, for which data can be used to better distinguish random coil and β -sheet structures by spectral response around 205-210 nm. Consistently, OmpF and OmpFG had CD spectra showing a distinct negative band at ~ 216 nm indicative of forming β -sheet structures with DLPC SUVs in the refolding buffer (Figure 5.4, black lines, —), while in the refolding buffer (1 M urea) without lipid, OMPs showed a less

distinct negative CD with a maximum at < 205 nm indicating a higher fraction of random coil structures (Figure 5.4, red lines, —). However, the CD spectra for OmpG are too noisy to distinguish between the folded and partially folded fractions, which may be due to impurities in the OmpG samples.

OMPs in this study all contain more than one Trp residues, hence it is possible to use fluorescence as a monitor of environmental change of Trp residues, which in turn is correlated to structural changes of the protein. The excitation wavelength for Trp fluorescence is 295 nm and the emission wavelength range monitored is from 300 nm to 450 nm. In Figure 5.5, the urea-denatured OmpF has a peak at 353 nm (blue line, —) and after mixing with DLPC SUVs it shifted to 332 nm with enhanced intensity, indicating the Trp residues changed to a more hydrophobic environment, most likely inserting into the lipid bilayers. The reduced urea concentration in the refolding buffer (1 M urea) results in a blue shift of OmpF fluorescence from 353 nm to 341 nm due to the change of solvent environment. In contrast to OmpF, OmpG and OmpFG with DLPC SUVs have less blue shift from the urea-denatured state, especially for OmpG. The resultant fluorescence peaks for OmpG in refolding buffer with and without lipid vesicle are at same wavelength (343 nm), although OmpG with DLPC SUVs has higher fluorescence intensity. OmpFG with lipid vesicles has a larger blue shift from its urea-denatured state than did OmpG (357 nm to 342 nm), indicating the Trps may have become more buried into a hydrophobic environment. These results are more related to the positions of Trps in the folded OMPs, which will be addressed in the following discussion section.



Figure 5.3 CD spectra of unfolded and refolded OMPs in 1 mm path cell. Unfolded OMPs in denaturation buffer (8 M urea, blue lines, —), partially folded OMPs in refolding buffer (1 M urea, red lines, —) and refolded OMPs with DLPC SUVs in refolding buffer (1 M urea, black lines, —).



Figure 5.4 CD spectra of folded and partially folded OMPs measured using shorter path length (0.5 mm). The partially folded OMPs in refolding buffer (1 M urea) without lipid are shown in red lines (—) and the folded fractions with DLPC SUV in refolding buffer are shown in black lines (—). Orange rectangles indicate the discrimination between β -sheet and random coil structures around 205 - 210 nm.



Figure 5.5 Fluorescence of folded, partially folded and unfolded OMPs. The unfolded OMPs in the denaturation buffer (8 M urea, blue lines, —), partially folded OMPs in the refolding buffer (1 M urea, red lines, —) without lipids and folded OMPs with DLPC SUV in refolding buffer (1 M urea, black lines, —).

Similar to OmpF, OmpG and OmpFG in the refolding buffer without lipid (red lines, —) has a blue shift that would be consistent with a change from its urea-denatured state to a partially-folded state due to the solvent exchange.

Although the CD and fluorescence results indicate OmpF and OmpG can all form some ordered structure by interacting with DLPC SUVs based on the SDS-PAGE, and CD results support them as well as OmpFG forming β -sheet structure in DLPC SUVs, the folding efficiency is not very high, which may be due to impurities in these protein samples. Therefore, it was necessary to utilize a method to purify these OMPs samples before refolding.

5.3.2 Purification of OMPs

The engineered OmpFG was constructed by combining the stable regions of OmpF and OmpG together. All three OMPs can be purified using an ion exchange column following the same protocol, ⁽²²⁵⁾ but they were eluted at different NaCl concentrations based on their distinct isoelectric points. As shown in Figure 5.6, after running through an ion exchange column and as determined by SDS-PAGE analysis, purified OmpF was eluted with 50-75 mM NaCl, OmpG was eluted with 200 mM NaCl and OmpFG was eluted with 140-160 mM NaCl. Other unwanted proteins were removed from these OMPs elution at different NaCl concentrations. The original OmpG was more impure than were OmpF and OmpFG, showing more unwanted protein fractions in lanes 2 and 3 (Figure 5.6). After purification, these unwanted proteins were all washed off the column at lower or higher NaCl concentrations. OmpF is relatively pure even without purification, only containing a very small amount of unwanted proteins, which can be washed out at high

NaCl concentration. The original OmpFG contains some other proteins with molar weights around 40 kD and 33 kD, which also were removed after purification.

5.3.3 Refolding of Purified OMPs using Lipid Vesicles and Detergents

In order to investigate the optimized folding conditions and increase the folding efficiency, the three purified OMPs studied here were diluted into lipid vesicles and detergent solutions for comparison. According to the SDS-PAGE results (Figure 5.7), after purification, OmpF formed trimer and dimer fractions in DLPC SUVs with relatively higher folding efficiency than did the unpurified samples (Figure 5.2), while in the refolding detergent (0.5 % DG and 0.2 % DM), it only formed a small fraction of trimer and no dimer was observed. By comparison to the heat modified denatured samples, OmpG was shown to form folded monomer in both DLPC SUV and OG refolding buffer with very high folding efficiency, only containing a minimal fraction of residual unfolded structures, in these cases enhanced by its relatively high purity. However, purified OmpFG still showed no mobility difference between unfolded (heat modified) and lipid or detergent solvated forms, which is consistent with OmpF monomer behavior, but again does not demonstrate folding.

The secondary structures of the purified OMPs interacting with DLPC SUVs were determined by CD spectra (Figure 5.8), and all these OMPs with DLPC SUVs (black lines, —) showed typical CD of forming β -sheet structures, especially for OmpG, which has well-defined band shape with larger amplitude than OmpF. This result is very consistent with the gel result (Figure 5.7) in that OmpG has the highest folding efficiency with lipid vesicles after purification.



Figure 5.6 Purification of OMPs determined by 12% SDS-PAGE. (a) Lane 1-5: marker, unpurified OmpF, OmpF elution fraction at 50 mM NaCl, OmpF elution fraction at 75 mM NaCl, other unwanted proteins. (b) Lane 1-3: marker, unwanted proteins eluted at 75 mM NaCl, unwanted proteins eluted at 250 mM NaCl concention. Lane 4-5: OmpG elution fractions at 200 mM NaCl. (c) Lane 1-4: marker, unpurified OmpFG, OmpFG elution fraction at 140 mM NaCl, elution fraction at 160 mM NaCl. All protein samples were heated at 95 °C for 5 min before loading onto the gel to appear at expected positons based on their molar weights.



Figure 5.7 Refolding of purified OMPs with lipid vesicles and detergents determined by 4-20 % gradient SDS-PAGE. The folded (F) and unfolded (U) OMPs were separated according to their heat modifiability. The unfolded OMPs in denaturation buffer (8 M urea) are in the lane 2, 7 and 12. Because there were not enough lanes in one single gel to show all the results, the lane 16-18 were from a different gel, which has been lined up with the gel containing lane 1 to 15.



Figure 5.8 CD spectra of folded and unfolded purified OMPs. Spectra for unfolded OMPs in the denaturation buffer (8 M urea) and folded fractions in the DLPC SUV are shown in blue lines (—) and black lines (—), respectively.

Fluorescence spectra of these purified OMPs in their urea-denatured and lipid-folded states are similar to those of the unpurified ones. As shown in Figure 5.9, OmpF has the largest blue shift (more than 20 nm) from unfolded state to folded state, followed by OmpFG, which is 12 nm and OmpG at 10 nm. Although the OmpG has a smaller shift than OmpFG, its intensity was highly enhanced after refolding by lipid vesicles, indicating its Trps stay in the more hydrophobic environment, and do not get quenched by other residues or solvent in the folding process, which is consistent with insertion into the lipid vesicle bilayer.



Figure 5.9 Fluorescence of unfolded and folded purified OMPs. Spectral for the unfolded OMPs in the denaturation buffer (8 M urea) and folded OMPs in DLPC SUV are shown in blue lines (—) and black lines (—), respectively.

5.3.4 Comparison of OMP Channel Conductivity by Dye Leakage Assay

The folded OMPs are expected to form a β -barrel channel in the outer membrane. Depending on the size and structure of the pores, the channel conductivities for transporting small molecules are different. A dye leakage experiment using lipid vesicles as a model membrane is a good method for study of the channel transporting property of OMPs *in vitro*. Carboxyfluorescein was used in this leakage assay, and its molar mass is 376 g/mol, which is small enough to pass through the porin channels. The DLPC SUVs with high concentration carboxyfluorescein (120 mM) inside had relatively quenched fluorescence and were isolated by a size exclusion column. OMPs were rapidly diluted into these lipid vesicles, after quickly mixing, the mixed samples including lipid vesicles without proteins were measured by fluorescence (Figure 5.10). In contrast to OmpG and OmpFG, OmpF with DLPC SUVs showed enhanced fluorescence immediately after quick mixing, but at that time the intensity of OmpG and OmpFG is similar to that obtained for lipid vesicles without any added protein.

After 19 hours incubation, the OMPs form stable pores in the lipid vesicles, and the trapped dye can leak out from the vesicles, but with varying efficiency. The leaked dye molecules become diluted and consequently have less self-quenching, which results in an enhancement of the overall quantum yield of the lipid-OMP sample. As shown in Figure 5.10, the fluorescence intensities were all enhanced after incubation, due to the leakage of dye molecules. Among the three OMPs, after incubation OmpF with DLPC has the most enhancement, followed by OmpFG and OmpG. By repeating the dye leakage experiment three times, the average of OmpF leakage is 77.6 %, higher than for OmpFG (48.8 %) and OmpG (34.6 %).



Figure 5.10 Carboxyfluorescein leakage experiment of OMPs folding into DLPC SUV. (a) Fluorescence of OMPs diluted into DLPC SUV, which have carboxyfluorescein trapped inside, after quick mixing. (b) Fluorescence intensities OMPs with DLPC SUV after 19 hours incubation. (c) The average leakage percentage of each OMP with lipid vesicles. The average of three repeats with standard deviations is shown.

5.4 Discussion

5.4.1 OmpFG may Follow the Folding Model of OmpF and OmpG

Several β -barrel proteins were previously reported to spontaneously fold into lipid bilayers or in detergent solutions *in vitro* without additional energy input. ^(223, 224, 238-242) It is impossible to establish a uniform folding model that all the OMPs will follow, because these OMPs require various conditions to optimize their folding efficiencies, including lipid and detergent properties, bilayer compositions, temperature, vesicle size and protein purity. However, since OmpFG is a combination of OmpF and OmpG, we started exploration of its folding conditions based on existing folding models for OmpF and OmpG.

Regarding the folding conditions of OmpF and OmpG, these follow some trends according to our observations. Both OmpF and OmpG prefer to fold in a thinner membrane rather than a thick one (Figure 5.1), since their folding efficiency significantly decreased in going from diC₁₂PC (DLPC) vesicles to diC_{18:1}PC (DOPC) vesicles. OmpF actually had no observed folded fraction in DOPC SUVs. This observation is consistent with previous reports that the preference of OMP to insert into thinner membrane is related to the localized structures of the proteins in the membrane.^(224, 243, 244) The average hydrophobic thickness of OMP is 23.7 \pm 1.3 Å, thus OMP can fold in diC₁₀PC and diC₁₂PC (DLPC) bilayers with a thickness of 26.5 Å and 30.5 Å, respectively, which is supported by calculational and experimental results.^(244, 245) Although the packing of DOPC is looser than for saturated lipids, which is caused by the double bonds in its hydrocarbon chains, the insertion of OmpF and OmpG is still inhibited apparently due to the thicker bilayers (~ 42 Å). DLPC bilayer is a proper membrane for insertion of both

OmpG and OmpF based on our observation of their forming folded fractions as determined by SDS-PAGE analysis (Figure 5.7). It should be noted that, in Figure 5.1, Figure 5.2 and Figure 5.7, the positions of OmpF dimer and trimer bands in the gel do not correspond to the molecular weight of the markers due to the different behaviors of folded OMPs in the gradient gel, which has also been reported in previous studies.⁽²²⁴⁾ The gradient SDS-PAGE is good for high resolution analysis of proteins having a large range of molar weights, in which the logarithm of protein molar weight is proportional to logarithm of distance traveled.⁽²⁴⁶⁾

In order to check the effect of lipid vesicle curvature on OmpF and OmpG refolding, both DLPC SUV (larger curvature) and LUV (smaller curvature) were used. The folding efficiency is slightly higher for OmpF and OmpG in SUV than in LUV (Figure 5.2). In SUV, more hydrophobic groups are exposed to proteins due to the larger curvature, which may lower the barrier for insertion of OMP if they are driven by the hydrophobic effect as asserted in a previous report.⁽²⁴⁷⁾ It should be noted that it is easier for OmpF to form dimers instead of trimers in SUV, which may be due to the limited capacity of small vesicles to take in large proteins. Overall OmpF and OmpG have higher preference for SUVs than LUVs.

OmpFG showed no heat modifiability behavior (difference in mobility on the gel) under any folding conditions, thus it is impossible to distinguish folded and unfolded fractions using the gels (Figure 5.2). However, we cannot conclude that OmpFG failed to fold into lipid just based on SDS-PAGE results, because it is possible that the monomeric OmpFG behaves similarly to the OmpF monomer, which also cannot be separated from unfolded fractions in the gel. In order to acquire more evidence to support our hypothesis, CD and fluorescence were applied to monitor the structural change of OmpFG with lipid vesicles.

All three OMPs with lipid vesicles formed some degree of secondary structures, most likely β -sheet components (Figure 5.3), by comparison to unfolded proteins in denaturation buffer. The extensive interactions between OMPs and lipid vesicles are further confirmed by fluorescence of Trp residues (Figure 5.5), the blue shift from unfolded state (—) to lipid-folded state suggesting the Trp residues enter into more hydrophobic environment by insertion into lipid bilayers. These spectroscopic results indicate OmpFG can interact with lipid vesicles to form stable β -sheet structures and imply that the Trp residues become buried inside the lipid bilayers.

It should be noted that the solvent environment can also play roles in promoting the folding of OMPs. As observed in just refolding buffer (1 M urea, 2 mM EDTA and 20 mM borate, pH 9.6), OMPs are partially folded showing some degree of structure formation in their CD spectra and having their fluorescence peak shift to shorter wavelength (Figure 5.3, Figure 5.4, Figure 5.5, Figure 5.8 and Figure 5.9). It should be noted that there is no significant peak shift between OmpG in lipid vesicles and just in refolding buffer, which is probably because most of Trp residues of folded OmpG are close to the edge of outer membrane (Chapter 1, Figure 1.14) and mostly exposed to solvent even if in the membrane. Thus the fluorescence peak positions are similar.

Other than lipid vesicles, OmpF and OmpG can also be refolded with the help of detergents (Figure 5.7), but only the trimer form of OmpF could be formed with detectable density on the gel in our detergent tests suggesting there may be the different folding mechanisms of OMPs in lipid vesicles and detergents. In the lipid bilayer, OMPs

went through a transition from urea denatured state to membrane absorbed and partially inserted state to folded β -barrel.^(248, 249) In the detergent solution, small detergent molecules first bind with hydrophobic regions of OMPs to make them water soluble, which enables them to form a partially folded β -sheet-rich intermediate, and then detergent environment allows all the strands to come together to form β -barrel structure.^(92, 96, 250) However, no separate folded fraction was observed for OmpFG in detergent, which is consistent with the lipid vesicle results.

Additionally, purity of the OMPs also affects folding efficiency. After removing unwanted proteins by purification, the folding efficiency increased significantly, especially for OmpG, which showed a much higher folded fraction in SDS-PAGE and obvious structural change with lipid vesicles as monitored with CD and fluorescence (Figure 5.7, Figure 5.8 and Figure 5.9).

Based on our spectroscopic observations, OmpFG appears to interact with DLPC SUVs to form an ordered β -sheet structure by inserting into the lipid bilayer. Very possibly, it forms a monomer which is very similar to the OmpF monomer, for which the heat modifiability is hard to be observed.

5.4.2 OmpFG can Form Pore with Relatively Higher Conductivity

Based on the prediction made by our collaborator, OmpFG is expected to form stable 16-stranded β -barrel having several specific characteristics: 1). It is proposed to form monomer instead of trimer due to the removal of L2 loop of OmpF, which provides a monomer-monomer interface. 2). By replacing the restriction region of OmpF (L3 loop) with the stable region of OmpG, the engineered OmpFG is designed to form a less restricted channel with higher conductivity. 3). OmpFG is predicted to form a larger channel than OmpG without gating behavior.

By comparing of the dye leakage assay used to determine average conductivity of pores formed by the three OMPs, OmpFG has a higher leakage fraction than OmpG, which should have a much narrower pore (Figure 5.10). The significant permeability of OmpFG provides good evidence for its successful refolding of OmpFG in lipid vesicles. Since no other bands were observed on the gel, this refolded structure is most likely a monomer. OmpF forms trimer and dimer fractions in lipid vesicles and has 78% leakage, which is more efficient than the OmpG or OmpFG. It also induces leakage immediately upon mixing with lipid vesicles, in contrast to the other OMPs, indicating a higher efficiency or a higher incorporation as a developed pore in the vesicle. Its trimer structure may enhance the permeability for the relatively small dye used by stabilizing the pore in the membrane. These conductivity results are consistent with our prediction based on modified protein structures, in which OmpFG should form pores with less constriction and higher conductivity than OmpG, but the OmpF comparison is not yet supportable. Perhaps tests with dyes of different sizes might help sort out this difference. A larger dye, Calcein (623 kD), failed to evidence leakage from the lipid vesicles in this study, presumably because its size is too large to pass through the OMP channels (good for substance < 600 kD). Other dyes with sizes between 400 to 500 kD could be used in future studies. On the other hand, OmpFG may have a relatively small fraction of folded form, which is an alternate explanation for the OmpF-OmpFG apparent difference in pore permeability.

5.5 Summary and Future Plan

The engineered OmpFG is designed to form a monomeric β -barrel channel with relatively high conductivity. Its folding in lipid vesicles was evidenced by CD, fluorescence and dye leakage assay. However the folding efficiency was hard to determine, since the folded and unfolded fractions of OmpFG cannot be separated by SDS-PAGE analysis. It is possible that the folded OmpFG monomer may have behavior similar to the OmpF monomer, both of which evidence no difference in mobility due to heat modifiability. However, the different behavior of trimeric OmpF in the dye leakage assay including rapid leakage and high conductivity, indicate the pore permeability for dye molecules may be related to structure of OMP (monomer, dimer or trimer), stability of barrel structure, size of dye molecule and folding efficiency of OMP in the membrane.

For better characterization, more tests are needed in future studies. Although the far-UV CD can provide secondary structural information which is related to the folding of OMP, it is not very sensitive to β -sheet. Instead of CD, FTIR might be useful in this study to determine the formation and organization of β -sheet structure. However, the utilization of FTIR for these samples requires dialysis or exchange of buffer to get rid of the 1 M urea in the refolding buffer, which would spectrally interfere with the amide I FTIR region of protein. Alternatively, a careful subtraction scheme might be able to compensate for the urea contribution.

In order to optimize the folding efficiency, other lipids or detergents could be used to refold OmpFG, such as lipids with similar length of hydrocarbon chains as DLPC but with different head groups and charged detergents that may interact with OMPs by electrostatic interaction, or lipid vesicles consisting of various lipids which exist in the cell outer membrane. Moreover, sonication of protein-lipid mixtures can provide premixing that may promote the insertion of OMPs into lipid bilayer.

Since the dye leakage assay could give more reliable results about the comparison of three OMPs after protein purification and optimization of refolding condition, other smaller (200 to 300 kD) and larger dyes (400 to 500 kD) can be used for characterizing the channel properties of OMPs, which may provide more detailed information about the size and stability of the channels.

Additionally, since OmpFG can be refolded with lipid vesicles or detergents, after incorporation in a membrane the channel properties can be characterized by single-channel recording experiments. This is the most direct method to provide detailed information about the kinetic behavior and permeability of channels, thus it has been widely used in ion channel and membrane protein studies.^(227, 251)

Appendix A. Expression of Mutant Forms of β -lactoglobulin

This unpublished research work was performed with the guidance and help of previous group members, Dr. Ning Ge and Mr. Martin Tibudan.

 β LG can undergo a β -sheet to α -helix secondary structure transition induced by lipid vesicles at neutral and low pH based on previous studies in Keiderling group. (80-82, 252) This process can be monitored by multiple spectroscopies: for the CD spectrum, the band shape of β LG changed from β -sheet to α -helix with addition of lipid vesicles (anionic lipid at neutral pH and zwitterionic lipid at low pH); the fluorescence intensity of β LG increased and the maximum wavelength had a blue shift when β LG was mixed with lipid vesicles, which indicate that at least one of the two Trp residues of β LG remains shielded in a hydrophobic environment; the polarized ATR-FTIR suggests the relative orientation between βLG and lipid bilayers, which is that βLG appears to insert its α -helical component into the lipid bilayer leave its β -sheet component lying on the surface of membrane. However, the exact orientation of βLG in the lipid bilayers is still unclear, and especially which segments undergo the β to α transition are unknown. Since the fluorescence of tryptophan is sensitive to the polar environment, it can be used to understand the location of different segments of lipid-bound protein in the lipid. There are two Trps in the β LG, Trp19 is buried in the hydrophobic core and Trp61 is exposed to the solvent in the native structure. In order to clarify the environment of each Trp in the lipid-bound β LG, we designed single point mutations of Trp19 and Trp61.

In order to get wild type of β LG expressed, we obtained strains containing constructed plasmids, which are *P.pastoris* GS115 β LG/pPIC9 and *E.coli* TG1, from Prof. Carl Batt at Cornell University.⁽²⁵³⁾ The pPIC9 vector (Invitrogen, San Diego, CA)

contains the alcohol oxidas (AOX1) promoter and a His⁺ selectable marker. The sequences and map of β LG/pPIC9 is shown in Figure A.1. The yeast *P. pastoris* strain has some advantages over *E. coli*, such as it can provide high yield of secreted proteins and eliminate the cell lysis step, therefore the use of yeast strain can simplify the expression and purification of β LG protein.

In order to eliminate fluorescence from one Trp at a time and change the environmental polarity of each Trp, the Trp19 and Trp61 were mutated into Leu or Ser. The designed mutagenic oligonucleotide primers (designed by my colleague) are shown in Table A.1. Each mutation was made on the isolated β LG/pPIC9 plasmid using PCR. The mutations were confirmed by DNA sequencing (UIC RRC). The PCR products (3 µl DNA) were then transformed into *Pichia* competent cells using EasyCompTM kit (Invitrogen, San Diego). The transformed competent cells were cultured on YPDS (yeast extract peptone dextrose medium with sorbitol) agar plates to yield a number of colonies.

The fresh single colony of *P.pastoris* was grown overnight in the 50 ml of glycerol complex medium (1% of yeast extract, 2% of peptone, 1.34% of yeast nitrogen base, 0.0004% biotin, 1% of glycerol and 100 mM potassium phosphate, pH7) at 30 °C with 200-250 rpm shaking until the OD₆₀₀ ~ 2. The cell pellets were harvested by centrifugation at 7000 rpm and were resuspended into BMMY medium (1% of yeast extract, 2% of peptone, 1.34% of yeast nitrogen base, 0.0004% biotin, 0.5% of methanol and 100 mM potassium phosphate, pH7) for large scale culture, until OD₆₀₀ ~ 1. Then the culture was induced by methanol to a final concentration of methanol as 0.5% for every 24 hrs. After 72 hrs, the protein was collected by centrifugation as supernatant.



Figure A.1 Restriction map of β LG/pPIC9. Adapted with permission from "High-level expression of bovine β -lactoglobulin in *Pichia pastoris* and characterization of its physical properties." Kim, T., Goto, Y., Hirota, N., Kuwata, K., Denton, H., Wu, S., Sawyer, L. and Batt, C. A. *Proein Engineering*, 1997, 10, 1339-1345.

Primer Name	Sequence 5' to 3'
W19S_5'	GTGGCGGGGGACTTCGTACTCCTTGGCC
W19S_3'	CACCGCCCCTGAAGCATGAGGAACCGG
W61S_5'	GGAGATCCTGCTGCAGAAATCGGAGAATGATGAGT
W61S_3'	CCTCTAGGACGACGTCTTTAGCCTCTTACTACTCA
W19L_5'	GTGGCGGGGGACTTTGTACTCCTTGGCC
W19L_3'	CACCGCCCCTGAAACATGAGGAACCGG
W61L_5'	GGAGATCCTGCTGCAGAAATTGGAGAATGATGAGT
W61L_3'	CCTCTAGGACGACGTCTTTAACCTCTTACTACTCA

Table A.1 Mutagenic oligonucleotide primers of β LG mutations.

The protein extraction was first filtered through 0.22 μ m filter to remove residual cell pellet. Then the purification of expressed β LG was performed using anion exchange column Q Ceramic HyperD F (Pall Life Science, Ann Arbor, MI). The filtered protein extraction was dialyzed against 20 mM phosphate buffer, pH7 and then loaded onto column. The column was washed by a gradient concentration of NaCl solution (0.1, 0.2, 0.3, 0.4, 0.5 and 1 M NaCl in 20 mM phosphate buffer, pH7). The eluted fractions were checked by SDS-PAGE. As shown in Figure A.2, the purified wild type of β LG was eluted at 0.3 M NaCl.

The β LG mutants including W19L, W19S, W61L and W61S were expressed and purified following the same protocol as wild type. The molecular weights of the mutants were confirmed by SDS-PAGE with comparison with β LG standard (Figure A.3). Additionally, the electrospray ionization mass spectrometry (MALDI) and mass spec amino acid digest sequencing (UIC RRC) were performed to confirm the success of the mutations on β LG.

Although we were able to express and purify the recombinant β LG and its mutants, these expressed proteins failed to fold into native structure in solution. As shown in Figure A.4, the CD spectra of the expressed recombinant β LG (blue line) showed a nonnative α -helical-like band shape and failed to yield a typical β -sheet band shape in comparison to that of commercial native β LG (red line). Similarly, the mutants were not able to fold into native β -sheet secondary structure in solution.

Based on the result of DNA sequencing of wild type β LG, there is an additional DNA sequence fragment (5 bps) at the end of the β LG DNA sequence in the recombinant plasmid, which may be a cause the failure of refolding.



Figure A.2 SDS-PAGE of standard β LG (Sigma) and eluted fractions after purification of recombinant β LG.



Figure A.3 SDS-PAGE of commercial β LG (Sigma) and mutants including purified W19L, W19S, W61L and W61S.



Figure A.4 CD spectra of commercial β LG (0.2 mg/ml, red line) and expressed recombinant β LG (0.2 mg/ml, blue line).

Appendix B

This part of work contains the basis of the study in Chapter 4, and was published, "On the DMSO-Dissolved State of Insulin: A Vibrational Spectroscopic Study of Structural Disorder", Dzwolak, D., Kalinowski, J., Johannessen, C., Babenko, V., Zhang, G. and Keiderling, T. A. *J. Phys. Chem. B*, **2012**, *116*, 11863-11871.

Upon dissolving in dimethyl sulfoxide (DMSO), the native insulin and insulin amyloid fibrils can convert into an identical disordered structural state based on IR spectral characteristics. Vibrational circular dichroism (VCD) can be used to investigate the DMSO-denatured state of insulin, because its band shapes and sign patterns developed are characteristic of conformation and are relatively independent of the frequencies of the underlying vibrational modes.

For VCD measurement, insulin from bovine pancreas (Sigma-Aldrich, USA) was dissolved at 3 wt% concentration in DMSO ("For Molecular Biology" grade from Sigma-Aldrich, USA) or DMSO- d_6 ("99.8 atom% D" grade from ARMAR Chemicals, Switzerland). Deuterated insulin was obtained through solvent exchange of 1 wt % insulin dissolved in D₂O ("99.8 atom % D" grade from ARMAR Chemicals, Switzerland), pD-adjusted to 1.9 (uncorrected readout of the pH-meter) with diluted DCl ("99 atom % D" grade from Aldrich). Each sample was sealed in a homemade transmission cell with CaF₂ windows separated by a 100 µm Teflon spacer. The spectra were recorded as an average of 8 scans over the amide I' region as described in Chapter 2.

The amide I/I' region VCD spectra of insulin in DMSO are shown in Figure 0.1.



Figure 0.1 VCD and FTIR absorption spectra of 3 wt % nondeuterated insulin in DMSO (solid line) and in DMSO- d_6 (dashed line), and of deuterated insulin in DMSO (dotted line) and in DMSO- d_6 (dashed-dotted line).

The VCD spectra are dominated by a negative couplet: positive at 1685 cm⁻¹ to negative, and more intense, at 1655 cm⁻¹. The VCD couplet shape is very similar to the spectral characteristics of peptides and polypeptides having local conformations approximating a left-handed 3₁-helix typically associated with the poly (L-proline) II conformation, and that of disordered and denatured proteins. However the component frequencies are much higher, again due to the absence of strong C=O H-bonds to the solvent in DMSO as compared to the more typical water solvent. Thus VCD suggests that DMSO-dissolved insulin is disordered with a high fraction of PPII like conformation. This VCD band shape is correlated with the central and higher frequency components of the IR band having been shifted up due to lack of H-bonding as described above. Moreover, the fact that the VCD signal is minimally affected by the deuteration of the backbone (unlike the IR absorption spectra) agrees well with the presence of PPII, as its VCD signature is known to be least affected by deuteration among all major protein conformations.

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