CHAPTER 7

Thesis summary and perspectives

OmpA is an ideal model for studying β-barrel membrane protein folding because it is one of the few integral membrane proteins that can spontaneously fold into micelles and vesicles from the fully denatured state in aqueous urea. A key question in β-barrel membrane protein folding is whether folding and membrane insertion are coupled or separable processes. The current OmpA model describes folding and insertion as coupled processes and that the four β-hairpins cross the membrane synchronously. Tertiary and secondary structure formation are also synchronous. However, there are still inconsistencies and unanswered questions with regard to this model.

The experiments described in this thesis utilized spectroscopic techniques to study the refolding and dynamics of OmpA. The overall goal was to provide further insight into the refolding mechanism by observing intermediates and transition states involved during the refolding process. Techniques used in this research included circular dichroism spectroscopy, steady-state and time resolved fluorescence spectroscopies, and fluorescence energy transfer (FET) kinetics. We used native tryptophan fluorescence as site-specific probes to monitor local changes in tryptophan environment.

Our first aim was to determine whether the C-terminus domain of OmpA affects the transmembrane structure. The two structures of OmpA have been solved using only the transmembrane N-terminal domain. We were curious to find out how the tryptophan environments were affected in the presence of the C-terminus. Steady-state and time-resolved fluorescence measurements of the tryptophan mutants in the full-length and truncated forms revealed that the microenvironment around the tryptophans are similar in both forms. These results support a picture in which the OmpA structure is not greatly perturbed by the presence of the C-terminus.

Fluorescence anisotropy decay kinetics revealed that W7 is the most flexible tryptophan position. This is possibly due to interaction with the C-terminal tail. Experiments using brominated lipids also did not show significant differences in quenching rates for the full-length and truncated mutants. Results confirmed previous reports that W7 fluorescence is the most quenched and suggest that this position is located closest to the hydrocarbon core. The fluorescence of W102 is the least quenched and is due to the orientation of this residue into the pore region.

The second aim was to determine whether the C-terminal plays a role in the folding mechanism. This question was investigated using fluorescence from single tryptophan mutants in a comparative study of full-length and truncated OmpA, where the C-terminus has been removed. Full-length mutants revealed that W102 fluorescence kinetics are the fastest and therefore the closest to reaching its folded environment. This result is consistent with reported results that W102 is one of the first tryptophans to insert. W7 fluorescence kinetics showed that this is the slowest tryptophan to reach its native environment. Removal of the C-terminal domain eliminates these subtle differences and therefore suggesting that the C-terminus may somehow play a role in the folding mechanism.

Investigations of the intramolecular distances during folding by fluorescence energy transfer kinetics from a tryptophan donor to a dansyl acceptor fluorophore were used to determine the time when the barrel ends come into contact. Results showed that these barrel ends develop native distances within 15 min of folding and do not change over the remaining course of folding. Future experiments may utilize a fast mixer to

measure kinetics on a shorter timescale. This will allow us to better resolve intermediates.

Experiments were also made at 15 ^{o}C, below the gel-liquid crystalline temperature of DMPC. In this lipid gel phase, OmpA adsorbs to the surface of the vesicles. FET kinetics revealed virtually no energy transfer taking place while in this species. This provides strong evidence that the adsorbed species is not an on-pathway folding intermediate. It may be possible that this species exists in equilibrium with the native structure since FET kinetics revealed that almost half the entire population remains unquenched. Our 15 ^{o}C steady-state fluorescence and circular dichroism also revealed that β-sheet formation occurs within the mixing dead time. The tryptophans are also immediately placed into a strongly hydrophobic environment. All of these results suggest that the 15 ^{o}C species is not an intermediate.

The studies of OmpA refolding described in this thesis lay the foundation for further in-depth studies using FET kinetics with donors and acceptors at other sites. In addition, the porin OmpF may be another protein candidate for FET kinetic studies. There are two native tryptophans in OmpF, located in the transmembrane regions. Comparison of FET kinetics between OmpA and OmpF may allow us to fully describe general characteristics of refolding and membrane insertion of β-barrel membrane proteins. OmpX is also another possibility since its structure resembles that of the truncated OmpA (Vogt & Schulz, 1999). Future studies may attempt to use unnatural amino acids to introduce fluorinated-tryptophan into OmpA. Fluoro-tryptophan has been shown to exhibit monoexponential decay kinetics (Winkler et al., 2006). This will allow

better resolution of the decay kinetics and a more straightforward analysis of FET kinetics.

Protein folding is a difficult field especially for the specific area of membrane protein folding due to the additional complication of lipids and detergents. A major question regarding protein folding in general is whether these *in vitro* studies are relevant to *in vivo* folding events. Before we can begin addressing and designing *in vivo* experiments, it is essential to build our tools and methods of investigations in a more controlled environment and develop a "toolbox" of folding models. When we finally are able to study *in vivo* protein folding, we can look in our toolbox and make intelligent and thoughtful interpretations about the mechanisms that we observe and measure.

REFERENCES

Vogt, J., and Schulz, G. E. (1999) The structure of the outer membrane protein OmpX from Escherichia coli reveals possible mechanisms of virulence, *Structure with Folding & Design 7*, 1301-1309.

Winkler, G. R., Harkins, S. B., Lee, J. C., and Gray, H. B. (2006) alpha-synuclein structures probed by 5-fluorotryptophan fluorescence and F-19 NMR spectroscopy, *Journal of Physical Chemistry B 110*, 7058-7061.

Kitty!

Yes, Ferret?

Let's get out of here! Chitter-chitter!

Woo-hoo! Meow!