Chapter VI

Conclusions and future perspectives
Biological membranes are dynamic and complex entities and the activity, functionality and structure of intrinsic membrane proteins are closely linked to the membrane environment. The biological membrane provides a unique anisotropic environment for protein function and folding, and lipid-protein interactions are critical to membrane protein function (Lee, 2004; Jensen and Mouritsen, 2004). It has become increasingly clear that membrane lipids play an active role in cellular physiology and membrane protein function, rather than only providing a passive barrier to the external environment. Specific lipid-protein interactions at the lipid-protein interface are essential for membrane protein function. The annular shell of lipid that surrounds the transmembrane region of membrane proteins is known to be required for membrane protein function (Jones and McNamee, 1988). The dynamics of this annular shell of lipids is distinct from bulk lipids (Ellena et al., 1983). Interestingly, such annular lipids have been resolved in a few high-resolution crystal structures (Fyfe et al., 2001; Lee, 2003; Palsdottir and Hunte, 2004). While it is still not clear if the presence of bound lipids in the crystal structure accurately represent interactions in the native environment (Lee, 2003), such structural details help to define the lipid-protein interface and specific interactions of residues at the protein interface with lipids. Due to the complexities associated with studying membrane protein structure, model systems provide convenient paradigms to study lipid-protein interactions. In this thesis, we chose the model ion channel gramicidin as a model membrane protein to study aspects of lipid-protein interactions, specifically, hydration and membrane thickness.

Gramicidins are linear pentadecapeptide antibiotics produced by the soil bacterium \textit{B. brevis} (Killian, 1992). Gramicidin forms prototypical ion channels specific for monovalent cations and has been extensively used to study the organization, dynamics and function of membrane-spanning channels (Killian, 1992; Andersen and Kopepe, 1992; Koepe and Andersen, 1996; Wallace, 2000; Miloshevsky and Jordan, 2004). Gramicidin serves as an excellent model for transmembrane channels due to its small size, ready availability and the relative ease with which chemical modifications can be performed.
(Greathouse et al., 1999). This makes gramicidin unique among small membrane-active peptides and provides the basis for its use to explore the principles that govern the folding and function of membrane-spanning channels in particular, and membrane proteins in general (Andersen et al., 1999; Martinac and Hamill, 2002).

It has become increasingly clear that water molecules mediate lipid-protein interactions (Ho and Stubbs, 1992; Essen et al., 1998; McAuley et al., 1999) and therefore the function of membrane proteins (Sankararamakrishnan and Sansom, 1995; Okada et al., 2002; Kouyama et al., 2004). Any alteration in the degree of hydration, particularly at the protein-lipid interface, could potentially lead to modifications of protein structure that could in turn modify its function. Water has been shown to act as a catalyst for hydrogen bond exchange in protein folding thereby acting as a 'foldase' (Xu and Cross, 1999). The membrane environment does not promote hydrogen bond exchange, making it possible to trap thermodynamically unstable conformations in the membrane environment (Killian et al., 1988; Arumugam et al., 1996). In such a scenario, it becomes important to study the effects of hydration on the conformation and dynamics of membrane proteins. Gramicidin is particularly suitable for such hydration studies due to its high surface to volume ratio and environment-sensitive conformational preference.

Biological and model membranes are not appropriate systems to explore the effects of hydration, since the controlled variation of water content is difficult in these systems. We therefore utilized reverse micelles as membrane-mimetic environments with controlled hydration (Luisi and Magid, 1986). Previous work from our group has shown that wavelength-selective fluorescence can be used to effectively monitor the changing dynamic hydration profile at the reverse micellar interface for a fluorescent lipid analogue, NBD-PE (Chattopadhyay et al., 2002) and the sole tryptophan residue of the amphipathic peptide, melittin (Raghuraman and Chattopadhyay, 2003). Since gramicidin is a multityrptophan peptide, it is possible that all the tryptophan residues would not be located at the reverse micellar interface. To obtain information about hydration-dependent solvent relaxation in the deeper regions of the reverse micelle we chose to use NBD-cholesterol as a probe. In NBD-cholesterol, the fluorescent NBD group is covalently attached to the flexible acyl
Chapter VI. Conclusions and future perspectives

chain of the cholesterol molecule and can function as a reporter of solvent dynamics in the deeper regions of the organized assembly. Earlier work has shown that the NBD group of NBD-cholesterol is localized in the deeper hydrocarbon region of organized assemblies (Chattopadhyay and London, 1987; Mukherjee and Chattopadhyay, 1996; Rawat and Chattopadhyay, 1999). Our results, presented in Chapter II, show that the rate of solvent relaxation (reorientation) varies with probe location in the reverse micellar assembly. Thus, while for interfacially located probes and peptides, REES decreases with increasing water content, REES for probes located deep in the acyl chain region of the reverse micellar assembly, increases. Such depth-dependent solvent relaxation effects could be important to interpret results from a heterogeneous population of reporter molecules in reverse micelles.

Gramicidin incorporated in reverse micelles was found to adopt the $\beta^{6,3}$ single-stranded conformation (Chapter III). Our results show that gramicidin tryptophans experience slow solvent relaxation in reverse micelles giving rise to REES, indicating that gramicidin tryptophans are located in a region of motional restriction in the reverse micelle, possibly at the reverse micellar interface. In addition, changes in fluorescence polarization with increasing excitation or emission wavelength reinforce that the gramicidin tryptophans are localized in motionally restricted regions of the reverse micelle. Interestingly, the extent of REES is found to be independent of the water content of the reverse micelle. This overall invariance of REES with water content in the reverse micelle was rationalized on the basis of environmental heterogeneity of gramicidin tryptophans in the reverse micellar assembly. As discussed above, the reverse micellar assembly exhibits depth-dependent solvent relaxation effects. Thus, while the extent of REES decreases with increasing water content for probes and peptides incorporated at the reverse micellar interface (Hof et al., 1997; Chattopadhyay et al., 2002; Raghuraman and Chattopadhyay, 2003), for probes located in the deep acyl chain region of the reverse micelle, REES was shown to increase with increasing water content. In other words, the rate of solvent relaxation (reorientation) varies with probe location in the reverse micellar assembly. In the background of these results, our result of relative invariance of the magnitude of REES with increasing water content...
content in case of gramicidin in AOT reverse micelles could be a result of heterogeneous environments around the gramicidin tryptophan residues. Since gramicidin is a multityptophan protein, the location of these tryptophan residues could be heterogeneous in the reverse micelle. While the carboxy terminal tryptophan (Trp-15) would occupy an interfacial position, the tryptophan residue at position 9 (Trp-9) would be placed in a relatively deep acyl chain region of the reverse micelle in the single stranded $\beta^{63}$ conformation. The overall variation in the extent of REES with increasing $w_0$ would then be dependent on the average of the variations with individual tryptophans and could result in the apparent insensitivity of the magnitude of REES to increasing water content for gramicidin in reverse micelles. Taken together, our results are significant in understanding the interaction of gramicidin with membrane-mimetic media under conditions of varying hydration.

If the overall invariance of REES with increasing water content is a result of an averaging of hydration dependent REES in different regions of the reverse micelle, it should be possible to test this proposal by utilizing single tryptophan analogues of gramicidin (in which three tryptophans are replaced by Phe). As discussed in Section I.2(ii), such synthetic single tryptophan analogues of gramicidin (Becker et al., 1991) form structurally equivalent channels with native gramicidin channels. However, as pointed out, the channel forming potency of these analogues is reduced as compared to the native sequence, possibly due to the presence of a population of non-conducting conformations. At low peptide/lipid ratios, the single stranded conformation of gramicidin is favored (Sawyer et al., 1990). Therefore for the low peptide/surfactant ratios used for these studies (Section III.2), single tryptophan analogues should form single-stranded $\beta^{63}$ helices with the tryptophan residues at a graded series of depths. In such a case, the Trp-15 analogue would exhibit hydration-dependent REES typical of probes at the reverse micellar interface and the Trp-9 analogue would behave like a deep probe in reverse micelles.

As a result of alternating L- and D- chirality, gramicidin can adopt a wide range of environment-dependent conformations (Urry, 1971; Veatch et al., 1974). Two major folding motifs for gramicidin in various media have been identified, the single-stranded
head-to-head helical dimers (the ‘channel’ form) and the double-stranded parallel or antiparallel intertwined helix (‘non-channel’ conformations). Interestingly, the initial conformation that gramicidin adopts when incorporated into membranes is dependent on its ‘solvent history’, i.e., on the nature of the solvent in which it was dissolved prior to incorporation in membranes (LoGrasso et al., 1988; Killian et al., 1988). However, in membranes the functional channel conformation is the ‘thermodynamically preferred conformation’ (Killian et al., 1988). We used the ‘solvent history’ of gramicidin to generate ‘channel’ and ‘non-channel’ conformations in membranes to monitor the non-channel and channel conformation utilizing sensitive fluorescence approaches (Chapter IV).

Our results show that REES of gramicidin tryptophans can be effectively used to distinguish conformations of membrane-bound gramicidin. The interfacially localized tryptophans in the channel conformation display REES of 7 nm while the tryptophans in the non-channel conformation exhibit REES of 2 nm which highlights the difference in their average environments in terms of localization in the membrane. This is supported by tryptophan penetration depth measurements using the parallax method and fluorescence lifetime measurements. Further differences in the average tryptophan microenvironments in the two conformations were brought out by fluorescence quenching experiments using acrylamide and chemical modification of tryptophan residues by NBS. In addition, we used the dual quenching method (Caputo and London, 2003b) to show that the gramicidin tryptophans in the channel conformation are on the average located in a shallow region of the membrane as compared to the relatively deep location in the non-channel conformation. These results are supported by quantitative measurements of the average depth of membrane penetration using parallax analysis. In summary, our results provide novel fluorescence-based approaches to monitor functional conformations of gramicidin.

The possibility of alternate, membrane bound conformations of gramicidin provides an opportunity to study the effects of the membrane environment on protein conformation (and function) using a well characterized model system. The hydrophobic thickness of the membrane is a fundamental property that can have an impact on transmembrane protein
Chapter VI. Conclusions and future perspectives

structure and function (Lee, 2004; Jensen and Mouritsen, 2004). Hydrophobic mismatch is a specific case of lipid-protein interaction that occurs when the hydrophobic thickness of the transmembrane region of a membrane protein does not match the unperturbed hydrophobic thickness of the membrane in which it resides. Importantly, the function of gramicidin channels has been directly related to membrane thickness (Elliott et al., 1983; Mobashery et al., 1997; Martinac and Hamill, 2002). We have examined the effects of hydrophobic mismatch on the conformation and organization of gramicidin in membrane bilayers of varying thickness utilizing the intrinsic conformation-dependent fluorescence of gramicidin. Our results, described in Chapter V, show that gramicidin remains predominantly in the channel conformation and gramicidin tryptophans are at the membrane interfacial region over a range of positive and negative mismatch conditions. However, gramicidin conformation tends to shift toward thermodynamically unfavorable non-channel conformations in very long bilayers. Interestingly, gramicidin is not excluded from the membrane even under conditions of extreme negative mismatch. To further probe the organization of gramicidin in mismatched membranes, we utilized tryptophan self-quenching to monitor the formation of mismatch-dependent aggregates in thicker bilayers. Our results show that gramicidin form self-quenched aggregates at ~2 mol% in long bilayers.

The experiments described in Chapter V utilized a series of saturated lipids to generate membranes of increasing thickness. We used saturated membranes in the gel phase so that hydrophobic mismatch would favor gramicidin conformational changes rather than membrane deformation due to the high cost of deformation of the saturated bilayers in the gel phase (see Section V.3). In fluid phase bilayers, however, the energetic cost of membrane deformation would be lower and therefore mismatch adaptation would primarily involve ordering/compression of the bilayer (de Planque et al., 1998; Weiss et al., 2003). A recent study reported the observation of closed gramicidin channels, i.e., monomeric gramicidin in the channel conformation, in long unsaturated bilayers in the fluid phase (Mo et al., 2004). In such a case, gramicidin tryptophan fluorescence would report the channel conformation, since it would not be sensitive to dimerization. However, preliminary
experiments in long unsaturated bilayers indicate aggregation of gramicidin (as determined by tryptophan self-quenching) at very low concentrations (~0.5-1 mol%). Further fluorescence spectroscopic analysis of gramicidin in these bilayers utilizing conformation-dependent fluorescence (as described in Chapter IV) and dual quenching analysis to assess tryptophan depths may be able to reconcile these results. In addition, a functional assay for ion transport by gramicidin channels in intact vesicles would help to determine if the predominant conformation of gramicidin in longer membranes is the functional dimer or monomeric gramicidin in the channel conformation. Functional assays that utilize potentiometric dyes such as carbocyanine (Sims et al., 1974) or ion binding dyes can be used to measure ion flux (Loew et al., 1983; Krishnamoorthy, 1986; Chen and Gross, 1995). It would be possible to use such dyes in a stopped-flow apparatus to measure ion fluxes in gramicidin containing vesicles.