Synopsis

Biological membranes are complex organized molecular assemblies of lipids and proteins that allow cellular compartmentalization and act as the interface through which cells communicate with each other and with the external milieu. Due to both lipid-protein as well as protein-protein interactions, the biological membrane constitutes the site of many important cellular functions. Further, membrane proteins are often targets for many pharmacological compounds. Knowledge of the structure and organization of membrane proteins therefore represents a major step toward understanding the function of membrane proteins. However, our understanding of the function of membranes in terms of lipid-protein interactions at the molecular level is limited by the lack of high-resolution three-dimensional structures of membrane-bound proteins and peptides. The main reason behind this lack of high-resolution membrane protein structures is often associated with the extreme difficulty in crystallizing membrane-bound proteins and peptides for diffraction studies. In order to understand lipid-protein interactions at the molecular level, both natural and synthetic membrane peptides have been used. This thesis is focused on exploring lipid-protein interactions in membranes using the hemolytic peptide melittin and a variety of membranes or membrane-mimetic systems utilizing spectroscopic approaches.

Melittin is a cationic, amphipathic, hemolytic peptide composed of 26 amino acid residues. It is intrinsically fluorescent due to the presence of a single tryptophan residue which has been shown to be crucial for its hemolytic activity and serves as a sensitive probe to monitor the interaction of melittin with membranes. The organization and dynamics of the tryptophan thus become important to understand the action of melittin on membranes. The amphipathic nature of melittin is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins. This has
resulted in melittin being used as a convenient model for monitoring lipid-protein interactions in membranes with the aim of understanding the general features of the structures of membrane proteins and their interactions with membrane lipids (Dempsey, 1990). It was shown earlier by our group that the sole tryptophan residue of melittin is located in a motionally restricted region in the membrane and the tryptophan environment is modulated in the presence of negatively charged phospholipids. A comprehensive review of melittin is provided in Chapter 1 (Section 1.3) of this thesis. The work presented in the rest of the thesis can be essentially subdivided into three major areas:

I. **Effect of Hydration on the Organization and Dynamics of Melittin**

Water plays a crucial role in the formation and maintenance of organized molecular assemblies such as proteins and membranes in a cellular environment. Knowledge of dynamics of hydration at the molecular level is, therefore, of considerable importance in understanding the cellular structure and function. In addition, water plays a crucial role in mediating lipid-protein interactions thereby controlling the functionality of membrane proteins. Biological and model membranes (liposomes), and membrane-mimetic systems such as micelles are not appropriate for exploring the effect of hydration on the stability and function of proteins since it is difficult to precisely control the water content in these systems. Reverse micelles represent a type of organized molecular assembly which offer the unique advantage of monitoring the dynamics of embedded molecules with varying degrees of hydration. This makes reverse micelles an appropriate system for hydration studies.

We have employed AOT reverse micelles as a membrane-mimetic system to monitor the effect of hydration on the organization and dynamics of melittin. This topic is presented in Chapter 2. Our results show that fluorescence parameters such as intensity, emission maximum and polarization of melittin incorporated in reverse
micelles of AOT in heptane are found to be dependent on [water]/[surfactant] molar ratio ($w_o$) of the reverse micelle. Time-resolved fluorescence measurements of melittin in AOT reverse micelles show a gradual reduction in mean lifetime with increasing $w_o$. More importantly, melittin in reverse micellar environment showed red edge excitation shift (REES) implying that localization of the peptide in reverse micelles results in considerable restriction to the reorientational motion of the solvent dipoles around the excited state fluorophore. Interestingly, the extent of REES was found to decrease with increasing $w_o$. Fluorescence polarization of melittin in reverse micellar environments was found to be wavelength-dependent. In addition, increasing hydration causes significant increase in helicity of melittin bound to reverse micelles. Taken together, these results provide information about the dynamics of melittin in environments of graded hydration and are relevant to dynamics of membrane-bound peptides under conditions of differential hydration which are more difficult to analyze experimentally.

II. Effect of Micellar Charge on the Conformation and Dynamics of Melittin

Electrostatic interactions play a crucial role in modulating and stabilizing molecular interactions in membranes and membrane-mimetic systems such as micelles. It is well established that electrostatic interactions are important in stabilizing lipid-protein interactions in membranes and in the process of membrane binding of proteins and peptides. In addition, electrostatic forces are important in determining the transmembrane orientation and topology of membrane-spanning $\alpha$-helices of integral membrane proteins. Melittin is known to interact selectively with negatively charged lipids and the presence of negatively charged lipids in the membrane has been shown to inhibit membrane lysis by melittin and this inhibition is enhanced with increasing surface charge density. In earlier work from our group, the microenvironment experienced by the sole tryptophan of melittin when bound to membranes of varying charge has been
monitored utilizing the wavelength-selective fluorescence approach. The results show that the tryptophan residue is located in a motionally restricted region in the membrane and the tryptophan environment is modulated by surface charge of the membrane which could be related to the difference in the lytic activity of the peptide observed in membranes of varying charge. We have monitored the change in the organization and dynamics of melittin bound to micelles of various charge types utilizing fluorescence and circular dichroism spectroscopy and these results are presented in Chapter 3. The sole tryptophan of melittin displays a REES of 3-6 nm when bound to anionic, nonionic and zwitterionic micelles. This suggests that melittin is localized in a restricted environment probably in the interfacial region of the micelles and this region offers considerable restriction to the reorientational motion of the solvent dipoles around the excited state tryptophan in melittin. Further, the rotational mobility of melittin is considerably reduced in these micelles and is found to be dependent on the surface charge of micelles. Interestingly, our results show that melittin does not partition into cetyltrimethylammonium bromide (CTAB) micelles due to electrostatic repulsion between melittin and CTAB micelles both of which carry positive charge. In addition, the fluorescence lifetime of melittin is modulated in micelles of different charge types. The lowest mean fluorescence lifetime is observed in the case of melittin bound to anionic sodium dodecyl sulfate (SDS) micelles. Circular dichroism spectroscopy shows that micelles induce significant helicity to melittin with maximum helicity being induced in case of melittin bound to SDS micelles. Fluorescence quenching measurements using the neutral aqueous quencher acrylamide show differential accessibility of melittin in various types of micelles. Taken together, these results show that micellar surface charge can modulate the organization and dynamics of melittin. These results could be relevant to understand the role of surface charge of membranes in the interaction of membrane-active, amphiphilic peptides with membranes.
III. Effect of Lipid Composition on the Organization and Dynamics of Membrane-Bound Melittin

It is well established that the organization of membrane-bound melittin is dependent on the physical state and composition of membranes. Lipids with polyunsaturated acyl chains are known to modulate a number of physical properties of membranes and play an important role in regulating the structure and function of membrane proteins. Interestingly, polyunsaturated lipids have also been shown to modulate the membrane-disruptive action of melittin. We have used melittin as a model peptide to address the influence of unsaturated lipids in modulating lipid-protein interactions. This topic constitutes Chapter 4 of the thesis. Our results show that fluorescence parameters such as intensity, emission maximum, polarization, lifetime and acrylamide quenching of melittin incorporated in membranes are dependent on the degree of unsaturation of lipids in membranes. Importantly, melittin in membranes composed of various unsaturated lipids show REES implying that melittin is localized in a motionally restricted region in membranes. Interestingly, the extent of REES was found to increase drastically in membranes with increasing unsaturation, especially when the lipids contained more than two double bonds. More importantly, increasing polyunsaturation in membranes inhibits the lytic activity of melittin. In addition, increasing unsaturation in membranes causes a considerable change in the secondary structure of membrane-bound melittin. Taken together, these results assume significance in the overall context of the role of unsaturated lipids in membranes in the organization and function of membrane proteins and membrane-active peptides.

The natural target membrane for the action of melittin is the erythrocyte membrane which contains high amounts of cholesterol. In addition, the presence of cholesterol in the membrane has been shown to inhibit the hemolytic activity of melittin. The organization of melittin in membranes containing cholesterol thus assumes significance. We have monitored the change in the organization and dynamics of melittin
induced by presence of cholesterol and this forms the subject matter of Chapter 5. Our results show that binding of melittin to membranes is inhibited in the presence of cholesterol. This is accompanied by an inhibition in the lytic activity of melittin as detected by calcein release assay and the extent of inhibition appears to be dependent on the concentration of membrane cholesterol. Interestingly, melittin is located in a motionally restricted environment in the membrane in the presence of cholesterol. The fluorescence emission maximum of membrane-bound melittin is shifted towards longer wavelength in the presence of cholesterol implying a change in polarity in the immediate vicinity of the sole tryptophan residue of melittin probably due to increased water penetration. This is supported by a concomitant reduction in fluorescence intensity, fluorescence lifetime and increased acrylamide quenching of melittin in the presence of high amounts of cholesterol. Membrane penetration depth analysis by the parallax method shows that the melittin tryptophan is localized at a relatively shallow depth in membranes containing cholesterol. The resonance energy transfer experiments using melittin/dehydroergosterol (DHE) as the donor/acceptor pair indicates the possibility of preferential interaction of melittin with membrane cholesterol. Overall, these results assume significance in understanding the interaction of melittin with membranes in general, and interaction with cholesterol-containing membranes in particular, with possible relevance to its interaction with the erythrocyte membrane.

Chapter 6 is a concluding chapter which contains an overall discussion of the work and also has suggestions for possible future work. I have chosen not to include a portion of my research work on (i) Reverse micellar organization and dynamics using a fluorescent lipid probe; (ii) Effect of urea on the organization and dynamics of Triton X-100 micelles; (iii) Organization and dynamics of NBD-labeled lipids; (iv) Influence of cholesterol and ergosterol on membrane dynamics; and (v) Effect of membrane curvature on the dynamics of membrane-bound molecules, since these topics are loosely related to the main focus of the thesis.