Chapter 6

Conclusions and Future Perspectives
Biological membranes are dynamic in nature and constitute the site of many important cellular functions due to the interplay between lipid-protein and protein-protein interactions (Shai, 2001). However, our understanding of the function of membranes in terms of lipid-protein interactions at the molecular level has been hampered by the lack of high-resolution three-dimensional structures of membrane-bound proteins and peptides. Further, contrary to the conception that lipids in membranes just act as a supporting matrix for membrane proteins, it is well recognized that the function of membrane proteins is affected by the presence of certain lipids in the membrane. For instance, cholesterol is shown to modulate the activity of Na\(^{+}\)-K\(^{+}\)-ATPase (Yeagle, 1985), receptors such as the oxytocin receptor (Gimpl et al., 1997), galanin receptor (Pang et al., 1999) and the serotonin\(_{1A}\) receptor (Pucadyil and Chattopadhyay, 2004), and cholesterol-dependent cytolysins (Giddings et al., 2003); PE acts as a molecular chaperone for the folding of Lactose permease (Bogdanov et al., 1999); SM is required for the insertion of Equinatoxin II, the most abundant hemolytic toxin isolated from sea anemone *Actinia equina* (Anderluh and Maček, 2002); polyunsaturated phospholipids are crucial for the stability and the functional activity of the visual pigment rhodopsin, a G-protein-coupled receptor (Litman and Mitchell, 1996; Polozova and Litman, 2000). The focus of this thesis is, therefore, to understand the molecular interaction of lipid-protein interactions in membranes under various conditions utilizing sensitive fluorescence-based approaches and CD spectroscopy. For this purpose, we chose melittin as a representative peptide for membrane-bound proteins and peptides.

Melittin is a cationic, amphipathic, hemolytic peptide isolated from the European honey bee, *Apis mellifera* and 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.
Interestingly, the tryptophan residue of melittin is localized in the interfacial region of membranes, a characteristic feature that is observed in the membrane proteins whose crystal structures are known (see Chapter 1, Section 1.1). In addition, melittin has been widely used as a convenient model for monitoring lipid-protein interactions in membranes for understanding the general features of the structures of membrane proteins and their interactions with membrane lipids. This is due to the amphipathic nature of melittin which is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins. It was shown by our group that the sole tryptophan of melittin is located in a motionally restricted interfacial region in the membrane and the tryptophan environment is modulated in the presence of negatively charged phospholipids (Chattopadhyay and Rukmini, 1993; Ghosh et al., 1997).

In general, the function of biological membranes with respect to lipid-protein interactions is governed by a combination of factors such as physical properties of membranes, hydration, electrostatics, and the presence of certain specific lipids in the membrane. We have addressed the effect of hydration on the organization and dynamics of melittin using AOT reverse micelles as a membrane-mimetic system (Chapter 2). 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 (Luisi and Magid, 1986). We have previously characterized the AOT reverse micelles utilizing the wavelength-selective fluorescence approach (Chattopadhyay et al., 2002). Our results presented in Chapter 2 show that the conformation and dynamics of melittin bound to reverse micelles is sensitive to varying degrees of hydration. Further, our results constitute the first report demonstrating that wavelength-selective fluorescence approach in general, and REES in particular, is sensitive to the changing dynamic hydration profile of an amphiphilic peptide. These results are significant especially keeping in mind the difficulty in precisely controlling the water content in biological membranes, model membranes and membrane-mimetic
systems such as micelles. It would be interesting to see whether the observed effects of hydration on the dynamics of melittin associate with the hemolytic activity of melittin.

Melittin, being a cationic amphipathic peptide, targets the membrane from the aqueous phase. In addition, biological membranes are composed of phospholipids which vary in their net charge (Gennis, 1989). The surface charge of the membrane is, therefore, a crucial factor in the binding of melittin with membranes. Interestingly, melittin is known to interact selectively with negatively charged phospholipids (Kleinschmidt et al., 1997; Lee et al., 2001) which affect the dynamics of membrane-bound melittin and inhibit the membrane-lytic action of melittin (Ghosh et al., 1997; Benachir and Lafleur, 1995). We have monitored the change in the conformation and dynamics of melittin bound to micelles of various charge types (Chapter 3). Overall, our results show that micellar surface charge can modulate the conformation and dynamics of melittin and these results could be relevant to understand the role of surface charge of membranes in the interaction of membrane-active, amphiphilic peptides with membranes.

Since biological membranes are composed of lipids which differ in their shape and size, it is rather impossible to identify the role of individual lipids required for the function of membrane proteins. Model membranes (liposomes) are appropriate systems to study the effect of specific lipid on the structure, stability and function of membrane peptides/proteins, since the complexity generally associated with natural membranes is not present and can be avoided in liposomes. We have used melittin as a model peptide to address the influence of unsaturated lipids in modulating lipid-protein interactions in membranes and the results are presented in Chapter 4. Interestingly, we show that the magnitude of REES obtained with melittin in membranes composed of more than two double bonds are higher than what is usually reported for membrane-bound tryptophan residues. Our results assume significance in the overall context of the role of unsaturated lipids in membranes in the organization and function of membrane-active peptides and membrane proteins such as rhodopsin.
In addition, we have monitored the change in the organization and dynamics of membrane-bound melittin induced by the presence of cholesterol (see Chapter 5). The study of the interaction of melittin with membrane cholesterol assumes significance since the natural target membrane for the action of melittin is the erythrocyte membrane which contains high amounts of cholesterol (Yeagle, 1985). Further, the presence of cholesterol in the membrane has been shown to inhibit the lytic activity of melittin (Benachir et al., 1997). The analysis of our results indicates that the dynamics of melittin is affected only by the presence of cholesterol at high concentration in the membrane. We attribute to the change in the hydration profile of membrane interfacial region induced by cholesterol at high concentration. Analysis of energy transfer experiments shows the possibility of preferential interaction of melittin with membrane cholesterol at low concentration. Overall, these results provide insight into the molecular mechanism of interaction of melittin with membranes in general, and interaction with cholesterol-containing membranes in particular.

Though melittin has been extensively used (including the work presented in this thesis) as a model peptide to understand lipid-protein interactions in membranes, the mechanism of the hemolytic activity of melittin is not completely understood. A number of questions can be asked to understand the mechanism of action of melittin in membranes. What is the organization and dynamics of the N-terminal region of melittin? Does the action of melittin in biological membranes depend on melittin's aggregation status? What is the role of cholesterol in the action of melittin in its natural target membrane, i.e., the erythrocyte membrane? Does cytoskeleton play a role in the hemolytic activity of melittin? Future studies could be aimed at the following approaches to answer some, if not all, of these questions. These studies not only help in understanding the mechanism of action of melittin but also would shed light in understanding the nature of lipid-protein interactions at a molecular level.
The sole tryptophan residue in the C-terminal region of melittin has been extensively used to obtain information on the conformation and dynamics of melittin in solution and its interaction with membranes. However, one disadvantage of this approach is that the information necessarily comes only from the C-terminal region of melittin and the information about N-terminal region is lacking. To overcome this problem and to obtain more comprehensive picture, covalent labeling of the amino terminal end or Lys-7 residue of melittin with a fluorescent NBD group can be quite useful. Apart from understanding the dynamics of N-terminal region of melittin, this approach would also help in monitoring the dynamics of the microenvironment of Lys-7, which has been shown to be crucial for the hemolytic activity of melittin (Blondelle and Houghten, 1991a). The NBD group is widely used to label proteins, peptides and lipids (Mukherjee et al., 1994). The NBD group has previously been shown by our group (Mukherjee and Chattopadhyay, 1996; Chattopadhyay and London, 1988; Chattopadhyay et al., 2002) and by others (Fery-Forgues, 1993) to be a very sensitive probe of the environment in which it resides. Since the sole tryptophan is located at the C-terminal region of melittin, the NBD-melittin analogues will provide an additional handle to monitor the conformation and dynamics of melittin in membranes under various conditions (e.g., different types of lipids, ionic strength, etc.) utilizing fluorescenc-based approaches and CD spectroscopy.

In addition, utilizing the environmental sensitivity of the NBD group of NBD melittin analogues would also be helpful in exploring the aggregation state of membrane-bound melittin. There is no consensus regarding the aggregation properties of membrane-bound melittin. This is due to the fact that the aggregation state of membrane-bound melittin is a complicated phenomenon and depends on several factors. These include concentration of peptide, ionic strength and phase state of the membrane in which melittin resides. Knowledge of the aggregation state of membrane-bound melittin is extremely important since this may shed light into the mechanism of action of this
hemolytic peptide and may help in understanding the structure-function relationship of other membrane proteins whose crystal structures are not known.

The aggregation property of the membrane-bound melittin can also be understood by monitoring the fluorescence resonance energy transfer between melittin and its photooxidized product in the membrane. Upon sufficient irradiation at 300 nm, the tryptophan residue of melittin is known to photooxidize and gives rise to its photooxidized product, N-formylkynurenine (NFK) melittin (Rao et al., 1990). The absorption maximum of NFK is around 340 nm and this provides the basis for efficient energy transfer with tryptophan/NFK as the donor/acceptor pair, since the fluorescence emission maximum of the tryptophan of membrane-bound melittin is ~335 nm.

As discussed above, the dynamics of membrane-bound melittin is affected by the presence of cholesterol only at high (>30 mol%) concentration which is interestingly the physiological concentration present in the human erythrocyte membrane (Lange et al., 1989). Surprisingly, we showed that melittin could preferentially interact with membrane cholesterol at low concentration. Whether the action of melittin involves selective targeting and partitioning of the peptide into cholesterol-rich domains of the target (erythrocyte) membrane represents an exciting possibility and remains to be explored. It would also be interesting to monitor the role of cholesterol in the hemolytic action of melittin in erythrocyte membranes by cholesterol depletion. Selective cholesterol depletion from the erythrocyte membranes can be achieved by treatment with methyl-β-cyclodextrin (MβCD), a compound that specifically extracts cholesterol from the membrane and leaving other lipids intact (Kilsdonk et al., 1995; Samuel et al., 2001). This approach, in combination with NBD-melittin and flow cytometry, would be extremely helpful in elucidating the functional importance of cholesterol in the hemolytic action of melittin.

Membrane phospholipid asymmetry is ubiquitous in the plasma membrane of many eukaryotic cells that modulates the mechanical stability of membranes (Manno et
The interaction of the cytoskeletal network with the cytoplasmic membrane (inner leaflet) controls the elasticity of the bilayer membrane. In addition, components of cytoskeletal network appears to localize to the cytoplasmic face of the membrane bilayer through the interaction with phosphatidylserine (MacDonald, 1993) present in the inner leaflet of the membrane. In addition, it has been reported that cytoskeletal proteins involve in the maintenance of dynamic (phase-state) asymmetry in erythrocyte membranes (Williamson et al., 1982). The inner leaflet association of cytoskeletal network with the erythrocyte membrane provides a possibility that this network could affect the interaction of melittin with erythrocyte membranes. This is interesting especially considering the fact that melittin interacts with the cytoskeleton in erythrocyte membranes (Hui et al., 1990). In addition, it has been reported that melittin does not adopt a fully transmembrane orientation (Wall et al., 1995; Bachar and Becker, 2000) and affects the inner leaflet of the bilayer more strongly than the outer leaflet (Bachar and Becker, 2000). The possibility of melittin-cytoskeletal protein interaction can be addressed and this could provide the insight into the mechanism of action of melittin in erythrocyte membranes.