Chapter 6

CONCLUSION AND FUTURE PERSPECTIVES
As has been discussed in this thesis, the dynamics exhibited by a given component of a large macromolecule such as a folded globular protein or an organized molecular assembly such as the biological membrane, is a function of its precise localization within the larger system. The focus of this thesis is the development and utilization of a set of approaches based on the red edge effect in fluorescence spectroscopy, which can be used to directly monitor the environment and dynamics around a membrane-bound fluorophore. Wavelength-dependent fluorescence (both steady state emission and polarization, as well as fluorescence lifetimes) can be effectively utilized in such systems to probe environment-induced motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. This approach makes it possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. This could prove to be a novel and powerful approach to probe environments in the vicinity of tryptophans (or other fluorescent residues, endogenous or introduced in a site-directed manner) in soluble proteins as well as in probes and proteins bound to membranes or supramolecular organizations such as protein-nucleic acid complexes. The advantages of this solvent relaxation based approach over the more conventional methods (such as fluorescence polarization) has already been pointed out in section 1.1 of this thesis.

Water plays a crucial role in the formation and maintenance of both folded protein and membrane architecture in a cellular environment. Knowledge of hydration at a molecular level is thus of considerable importance in understanding the cellular structure and function (Crowe & Crowe, 1984; Rand & Parsegian, 1989; Ho & Stubbs, 1992; Ho et al., 1994). As has been pointed out earlier, the red edge effect is based on the change in fluorophore-solvent interactions in the ground and excited states brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which
solvent molecules reorient around the excited state fluorophore. Since for most biological systems, the ubiquitous solvent is water, the information obtained in such cases will come from the otherwise ‘optically silent’ water molecules. This makes the use of REES and related techniques extremely useful in biology since hydration plays a crucial modulatory role in a large number of important cellular events (Crowe & Crowe, 1984; Rand & Parsegian, 1989; Ho & Stubbs, 1992; Ho et al., 1994).

The biological membrane is a highly organized molecular assembly, largely confined to two dimensions, and exhibits considerable degree of anisotropy along the axis perpendicular to the membrane plane (Seelig, 1977; Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992; White & Wimley, 1994). This not only results in the anisotropic behavior of the constituent lipid molecules, but more importantly, the environment of a probe molecule becomes very much dependent on its precise localization in the membrane. The biological membrane, with its viscous interior (Cone, 1972; Poo & Cone, 1974), and distinct motional gradient along its vertical axis, thus provides an ideal system for the utilization of REES to study various membrane phenomena. The use of this technique becomes all the more relevant in view of the fact that no crystallographic database for membrane-bound probes and proteins exists to date, due to the inherent difficulty in crystallizing such molecules. Although many important functions are associated with cell membranes, our understanding of these processes at the molecular level is limited by the lack of high resolution three dimensional structures of membrane-bound molecules. It is extremely difficult to crystallize membrane-bound molecules for diffraction studies. Only a few years back was the first complete x-ray crystallographic analysis of an integral membrane protein successfully carried out (Deisenhofer et al., 1985).

The results presented in this thesis raises further questions that could be addressed
using similar approaches. For example, the restricted environment experienced by the functionally important tryptophan residues at the peptide-lipid interface of the gramicidin channel is demonstrated in chapter 4, and it is shown the channel tryptophans can be grouped into at least two classes experiencing very different microenvironments. However, it is still not possible to assign specific roles to each of these tryptophans. It will indeed be very interesting if mutants of gramicidin can be synthesized, lacking one or more of these tryptophan(s) in various combinations. A systematic study of these mutants along the lines carried out with native gramicidin in chapter 4 as well as depth analysis using the parallax approach (Chattopadhyay & London, 1987) should prove to be useful in evaluating the role of individual tryptophans in maintaining the ion channel structure and function. That channel characteristics exhibited by such mutants are very different from the native channel has already been demonstrated by elegant electrophysiological experiments by Andersen and colleagues (Becker et al., 1991; Fonseca et al., 1992). The significance of this study in terms of functioning of the gramicidin channel is also brought out by the fact that the cation conductivity of the channel decreases upon substitution of one or all of the tryptophan residues by phenylalanine, tyrosine or naphthylalanine (Bamberg et al., 1976; Heitz et al., 1982; Prasad et al., 1983; Trudelle & Heitz, 1987; Daumas et al., 1989; Becker et al., 1991; Fonseca et al., 1992), and also upon ultraviolet irradiation or chemical modification of the tryptophan rings (Busath & Waldbilling, 1983; Jones et al., 1986; Strassle et al., 1989). In fact, it has been proposed that the tryptophan dipoles facilitate ion entry into the gramicidin channels by aligning with the lipid head group electric field and producing a contrary field that reduces the surface potential of the membrane, which under normal circumstances inhibits ion entry into the bilayer (Busath, 1993).

Further, in has been suggested that the tryptophans in the gramicidin channel, which are localized at the membrane interface, are motionally restricted probably due to hydrogen
bonding with either the carbonyl groups of the neighboring lipids, or with the interfacial water molecules. Similar REES experiments can be performed with gramicidin channels incorporated into membranes made of ether lipids (as against the more commonly used ester lipids, such as DOPC). If the lipid carbonyls of DOPC were the major hydrogen bond acceptors, then no such carbonyls will be available in case of ether lipids, and this may show up as a reduction in the magnitude of REES, signifying a less motionally restricted environment in the absence of hydrogen bonding with the lipid carbonyls. Similarly, if the experiment with gramicidin channels in DOPC vesicles is done in deuterium oxide (D$_2$O) rather than in normal water (H$_2$O), the magnitude of REES could throw light on the different reorientation rates of D$_2$O compared to H$_2$O. Such an increased magnitude of REES will indicate a specific interaction (hydrogen bonding) of the tryptophan residues of the gramicidin channels with interfacial water molecules. Such experiments carried out in membranes made of either ether or ester lipids will help assess the relative contributions of hydrogen bonding of the tryptophan residues with the lipid carbonyls vs. the interfacial water molecules in maintaining the channel conformation of gramicidin.

A lot of evidence accumulated over the past few years points out that extensive transverse as well as lateral regionalization of both proteins and lipids, which can be described in terms of macro- and microdomains, are common features in many biological membranes (Curtain et al., 1988; Edidin, 1992). Macrodomains correspond to large membrane areas of at least few tenths of a mm in surface and have been identified in the plasma membranes of certain specialized cells with highly regionalized functions such as spermatozoa, eggs, epithelia, and erythrocytes (Simons & Wandinger-Ness, 1990; Wolf et al., 1990; Rodgers & Glaser, 1991; Mostov et al., 1992). Microdomains, on the other hand, extend from the submicron to the molecular scale, and are still poorly understood.
(Melchior, 1986). Thus, one of the most challenging problems in membrane biology today is to get a clear picture of lipid and protein organization and mobility at a microscopic and even molecular level. Such microdomains often differ in fluidity from the bulk membrane, and according to the current working hypothesis, these domains originate from (i) presence of a protein lattice within the membrane, preventing lipid molecules from diffusing freely in the plane of the membrane, (ii) specific and non-specific interactions between lipid and protein molecules, and (iii) mismatch between the hydrophobic thicknesses of transmembrane proteins and the surrounding lipid bilayer (Mouritsen & Bloom, 1984; Yechiel & Edidin, 1987; Dupou et al., 1988; deBony et al., 1989; Lavergne & Joliot, 1991). Since the motional restriction experienced by a fluorophore in these domains will differ from both the bulk membrane, as well as within different coexisting domains, a parameter such as REES promises to be an effectively tool to study the motional characteristics of such domains, which may have important functional implications. For such a study, it will be necessary to have probes that preferentially partition into either the more mobile or the more motionally restricted domain. Many such probes are already known in the literature. For example, the trans isomer of a fluorescence probe, parinaric acid, has a partition coefficient of 4.2 preferring the gel phase, while cis parinaric acid has a partition coefficient of about 1.0, distributing fairly evenly between the gel and fluid phases (Sklar et al., 1979; Hudson & Cavalier, 1988). Similarly, the short chain lipophilic carbocyanine dye DiIC<sub>11</sub>(3) has been shown to have a substantial fluid phase preference (~ 6:1), whereas DiIC<sub>6</sub>(3) is predominantly distributed in the gel phase (~ 1:10) (Spink et al., 1990). Any results obtained from such studies will have tremendous functional implications. For example, the glycosylphosphatidylinositol (GPI)-anchored proteins, in which the carboxy-terminal peptide sequence is replaced by a GPI moiety that serves as a membrane anchor, have been shown to be clustered into microdomains at the cell surface
over specific membrane invaginations called caveolae, in association with caveolin (a cytoplasmic caveolar coat protein), as well as cholesterol and glycolipids (Sargiacomo et al., 1993). Such clustering has been shown to depend upon the presence of cholesterol in the membrane (Rothberg et al., 1990). It has been shown that such clustering of GPI-anchored proteins such as Thy-1 can lead to the generation of intracellular signals. This is thus one situation where a microdomain, perhaps containing a specialized lipid composition, is thought to play an important role in cell physiology.

Similarly, transbilayer asymmetry giving rise to leaflets that can be considered as specialized domains having differential functional properties, has been observed in various cells, the most well known example being that of the erythrocyte (Verkleij et al., 1973). For example, the outer leaflet of erythrocytes are specifically enriched in phosphatidylcholine and sphingomyelin, whereas the inner leaflet is enriched in phosphatidylethanolamine. Further, phosphatidylserine is found exclusively in the inner leaflets in the membranes of these cells. Such asymmetry is often induced in model membranes as well, depending on the lipids being used, as well as the size of the vesicles, and resultantly, the radii of curvature of the outer vs. the inner leaflets of these membranes (Sheetz & Chan, 1972; Chrzeszczyk et al., 1977; Schuh et al., 1982). The motional characteristics in such systems can be studied by labeling the membranes with head group labeled probes such as NBD-PE, and selectively reducing the NBD labels from the outer leaflet using the water soluble reducing agent dithionite, $\text{S}_2\text{O}_4^{2-}$ (McIntyre & Sleight, 1991; Langner & Hui, 1993; Balch et al., 1994). The resulting vesicles will have fluorescence originating exclusively from the NBD groups located in the inner leaflet. These asymmetric vesicles are stable for hours, since flip-flop of lipids is slow in general (Kornberg & McConnell, 1971) and more so for polar fluorophores such as NBD-PE (McIntyre & Sleight, 1991). In addition, the doubly charged dithionite ion permeates very
slowly to the inner leaflet (Langner & Hui, 1993). Wavelength-dependent fluorescence studies performed on these ‘asymmetrically fluorescent’ vesicles will provide valuable insight into the dynamics of the inner leaflet (which for small vesicles, could be very different than that of the outer leaflet). For preferential reduction of the fluorescence from the inner leaflet, the vesicles have to be prepared in buffer containing dithionite, quickly passed through a gel filtration column, and resuspended in buffer without dithionite. REES and time resolved fluorescence studies of these vesicles will selectively reflect the dynamic properties of the outer leaflet. In case of experiments with intact cells, fluorescent derivatives of those lipids that are selectively found in one of the leaflets can be used.

In conclusion, the phenomenon of red edge effect can be effectively utilized as a novel and very powerful tool to address various questions in membrane biology. Nevertheless, the results presented in this thesis indicate that fluorescence studies in motionally restricted systems should be carried out with caution. Red edge effects should be taken into consideration if such fluorophores are excited at the red edge of the absorption spectra rather than at the absorption maxima. Failure to do so in studies such as energy transfer could lead to significant errors. In fact, it has been recently observed that there is a wavelength-dependent variation in the measured location (depth) of fatty acyl attached probes in the membrane (Abrams et al., 1992). In general, it was found that there is a decrease in the apparent depth of the fatty acyl attached probes both at longer excitation wavelengths and at longer emission wavelengths.

Another potential complication due to red edge effects could arise while interpreting emission characteristics and fluorescence lifetimes of the tryptophan residues in proteins and peptides. The excitation maxima of tryptophan and tyrosine residues are very close (280 and 275 nm, respectively). In order to selectively excite tryptophan residues, therefore, proteins are often excited at 295 nm (red edge of the tryptophan absorption band,
having minimal interference from the tyrosine excitation), instead of 280 nm, which is the mean excitation wavelength for tryptophan (Teale, 1960; Longworth, 1971). If all the tryptophans of the protein are well exposed to the solvent, it is unlikely that the red edge effect will be operative in fluid solutions. In such a case, excitation at 295 nm will not introduce any artifacts in either the emission maximum or the lifetime of the tryptophan residues. However, tryptophans in polar yet restricted environments (e.g., buried or membrane-bound tryptophans having water or other amino acid side chains or lipid carbonyl dipoles in its immediate vicinity) may well be subject to this effect. Under such conditions, excitation of the protein at 295 nm could introduce a red shift in its emission maximum, as well as a reduction in its mean lifetime, both of which may be incorrectly interpreted as tryptophan residues being more exposed to the bulk aqueous phase than they really are. Thus, it is important to consider the choice of wavelengths in such experiments.

While used with the above-mentioned caution in mind, wavelength-dependent fluorescence (both steady state emission and polarization, as well as time resolved fluorescence) offers a convenient handle to probe environment-induced motional restriction imposed on the solvent molecules in the immediate vicinity of a fluorophore in complex biological systems. However, the lack of a suitable fluorophore often makes it difficult to monitor dynamics in a region of interest. Fortunately, recent advances in molecular biological techniques have made it possible to incorporate (or substitute) endogenous labels such as tryptophan in regions of choice in soluble (Gopal et al., 1994) as well as integral membrane proteins (Menezes et al., 1990). A major limitation in working with multityrptophan proteins is that the analysis of fluorescence is often complicated due to the complexity of fluorescence processes in such systems (Eftink, 1991; Chattopadhyay & McNamee, 1991). Site-specific incorporation of extrinsic probes, very recently accomplished by using unnatural amino acid mutagenesis (Cornish et al., 1994), should
help avoid this complication. Wavelength-selective fluorescence studies, in conjunction
with these powerful molecular biological approaches, could prove to be a novel and
extremely powerful tool to probe environments in the vicinity of uniquely localized
tryptophans (or other fluorescent residues, endogenous or introduced in a site-directed
manner) in soluble proteins as well as in probes and proteins bound to membranes or
supramolecular organizations such as protein-nucleic acid complexes.