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NOVEL INSIGHTS INTO MEMBRANE PROTEIN STRUCTURE AND DYNAMICS UTILIZING THE WAVELENGTH-SELECTIVE FLUORESCENCE APPROACH

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Wavelength-selective fluorescence comprises 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 fluorophore in a complex biological system. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises from slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes 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. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases will come from the otherwise 'optically silent' water molecules. This makes REES and related techniques extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events including lipid-protein interactions and ion transport. The application of the wavelength-selective fluorescence approach as a powerful tool to monitor organization and dynamics of membrane proteins and peptides is discussed in this review.

Key Words: Wavelength-Selective Fluorescence Approach; Red Edge Excitation Shift (REES); Membrane Proteins and Peptides; Tryptophan Fluorescence; Hydration Dynamics; Solvent Dipole Relaxation

1 Introduction

Biological membranes are complex 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. The biological membrane constitutes the site of many important cellular functions. However, 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 proteins or peptides for diffraction studies. Although the first complete X-ray crystallographic analysis of an integral membrane protein was successfully carried out a number of years back[1], the number of membrane proteins whose X-ray crystal structures are known is still very small and represent only ~0.2% of all solved protein structures[2]. This is in spite of the fact that about 30-40% of all proteins are integral membrane proteins[3]. The great disparity between our understanding of soluble proteins and membrane proteins is a consequence of many practical problems of working with membrane proteins. Even high resolution NMR methods have limited applications for membrane-bound proteins and peptides because of slow reorientation times in membranes[4].

Due to the inherent difficulty in crystallizing membrane proteins, most structural analyses of such molecules have utilized other biophysical techniques with an emphasis on spectroscopic approaches. Spectroscopic techniques which provide both structural and dynamic information therefore become very useful for analyses of

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membrane proteins. Fluorescence spectroscopy represents one such approach and is widely used in analysis of membrane protein structure and function. The advantages of using fluorescence techniques are intrinsic sensitivity, suitable time scale, non-invasive nature, and minimum perturbation [5-8]. This review is focussed on the application of a novel approach, the wavelength-selective fluorescence approach, as a powerful tool to monitor the organization and dynamics of, membrane proteins and peptides.

Wavelength-selective fluorescence comprises 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 fluorophore in a complex biological system. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES)[8-10].This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises from slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. This approach therefore allows one to probe the mobility parameters of the environment itself (dynamics of solvation) represented by the relaxing solvent molecules using the fluorophore merely as a reporter group. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases will come from the otherwise 'optically silent', water molecules. This makes REES and related techniques extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events. The application of the wavelength-selective fluorescence approach to membranes and membrane-mimetic systems has been recently reviewed[8,9].

2 Red Edge Excitation Shift (REES)

In general, fluorescence emission is governed by Kasha's rule which states that fluorescence normally occurs from the zero vibrational level of the first excited electronic state of a molecule[11,12]. It is obvious from this rule that fluorescence should be independent of the wavelength of excitation. In fact, such a lack of dependence of fluorescence emission parameters on excitation wavelength is often taken as a criterion for purity and homogeneity of a molecule. Thus, for a fluorophore in a bulk non-viscous solvent, the fluorescence decay rates and the wavelength of maximum emission are usually independent of the excitation wavelength.

However, this generalization breaks down in case of polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases, that is, when the mobility of the surrounding matrix relative to the fluorophore is considerably reduced. This situation arises because of the importance of the solvent shell and its dynamics around the fluorophore during the process of absorption of a photon and its subsequent emission as fluorescence (see later). Under such conditions, when the excitation wavelength is gradually shifted to the red edge- of the absorption band, the maximum of fluorescence emission exhibits a concomitant shift toward higher wavelengths. Such a shift in the wavelength of maximum emission toward higher wavelengths, caused by a corresponding shift in the excitation wavelength toward the red edge of the absorption band, is termed the Red Edge Excitation Shift (REES)[8,13,15]. Since REES is observed only under conditions of restricted mobility, it serves as a reliable indicator of the dynamics of fluorophore environment.

The genesis of REES lies in 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[14,16,22]. For a polar fluorophore, there exists a statistical distribution of solvation states based on their dipolar interactions with the solvent molecules both in the ground and excited states in order to minimize the energy of the given state. Since the dipole moment of a molecule changes upon excitation[23], the solvent dipoles have to reorient around this new excited state dipole moment of the fluorophore, so as to attain an energetically favourable orientation. This readjustment of the dipolar interaction of the solvent molecules with the fluorophore essentially consists of two components. First, the redistribution

of electrons in the surrounding solvent molecules because of the altered dipole moment of the excited state fluorophore, and then the physical reorientation of the solvent molecules around the excited state fluorophore. The former process is almost instantaneous, i.e., electron redistribution in solvent molecules occurs at about the same time scale as the process of excitation of the fluorophore itself (10^{-15} sec). The reorientation of the solvent dipoles, however, requires a net physical displacement. It is thus a much slower process and is dependent on the restriction to their mobility as offered by the surrounding matrix. More precisely, for a polar fluorophore in a bulk non-viscous solvent, this reorientation time τ_S is of the order of 10^{-12} sec, so that all the solvent molecules completely reorient around the excited state dipole of the fluorophore well within its excited state lifetime τ_F which is typically of the order of 10^{-9} sec[a]. Hence, irrespective of the excitation wavelength used, all emission is observed only from the solvent-relaxed state. However, if the same fluorophore is now placed in a viscous medium, this reorientation process is slowed down such that the solvent reorientation time (τ_S) is of the order of 10^{-9} sec or longer. Under these conditions, excitation by progressively lower energy quanta, *i.e.,* excitation wavelength being gradually shifted toward the red edge of the absorption band, selectively excites those fluorophores which interact more strongly with the solvent molecules in the excited state. These are the fluorophores around which the solvent molecules are oriented in such a way as to be more similar to that found in the solvent-relaxed state. Thus, the necessary condition for giving rise to REES is that a different average population of fluorophore is excited at each excitation wavelength and, more importantly, that the difference is maintained in the, time scale of fluorescence lifetime. As discussed above, this requires that the dipolar relaxation time for the solvent shall be comparable to or longer than the fluorescence lifetime so that fluorescence occurs from various partially relaxed states. This implies a reduced mobility of the surrounding matrix with respect to the fluorophore.

The essential criteria for the observation of the red edge effect can therefore be summarized as follows: (i) the fluorophore should normally be polar so as to be able to suitably orient the neighbouring solvent molecules in the ground state; (ii) the solvent molecules surrounding the fluorophore should be polar; (iii) the solvent reorientation time (τ_S) around the excited state dipole moment of the fluorophore should be comparable to or longer than the fluorescence lifetime (τ_F) and (iv) there should be a relatively large change in the dipole moment of the fluorophore upon excitation. The observed spectral shifts thus depend both on the properties of the fluorophore itself (i.e., the vectorial difference between the dipole moments in the ground and excited states), and also on properties of the environment interacting with it (which is a function of the solvent reorientation time τ_S). It has previously been shown by us that for 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labelled phospholipids incorporated into model membranes, a dipole moment change of ~4 D upon excitation is enough to give rise to significant red edge effects[23]. A recent comprehensive review on the red edge effect is provided by Demchenko[24].

3 The Two State Model

The two state model provides a simple conceptual framework within which one can consider the phenomenon of solvent relaxation[18,25-27]. This model assumes that fluorescence emission proceeds from two discontinuous or discrete states (see Fig. 1): the initial or Franck-Condon (F) state and the final or relaxed (R) state. Excitation at the maximum (center) of the absorption band (λ_C), initially yields the Franck-Condon excited state around which solvent reorientation has not taken place. This initially excited state F can then decay to a completely relaxed state R where solvent reorientation around the excited state fluorophore is complete, with a characteristic relaxation time τ_S. The rate of this relaxation is determined by both general and specific interactions between the fluorophore and the surrounding solvent molecules and the rate at which these interactions are modified in response to the newly created excited state dipole

a Use of a single parameter τ_S to describe the relaxation of solvent molecules is a first order approximation since a set of relaxation times would exist in real systems.[7] However, such an approximation is often made to make the relaxation model simple. Thus, τ_S may be considered as a simple effective parameter characterizing the solvent relaxation process.

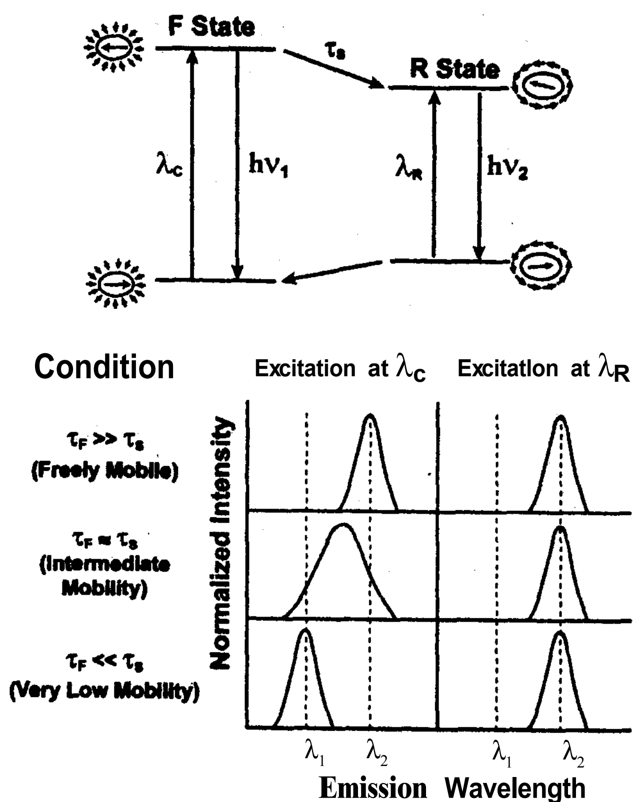

Fig. 1 A schematic representation of the two state model of solvent relaxation in terms of energy levels (top) and fluorescence emission spectra (bottom). F and R states refer to the Franck-Condon (or initial) and relaxed (or final) excited states. τ_S is the reorientation time of the solvent molecules and τ_F is the lifetime of the fluorophore. The large arrows represent the dipole moment vectors of the fluorophore and the small arrows denote those of the solvent molecules surrounding the fluorophore. λ_C and λ_R are the respective wavelengths associated withdirectexcitation of the Franck-Condon and relaxed states. See text (Section 3) for other details.

moment. If we denote the direct excitation of the F state as λ_C and such excitation of the R state as λ_R (where $\lambda_R > \lambda_C$), then there could be three possibilities (Fig. 1). If the rate of solvent reorientation is much faster than the fluorescence lifetime ($\tau_F >> \tau_S$), the solvent molecules have enough time to reorient before the fluorophore emits. Therefore, irrespective of the excitation wavelength used (λ_C or λ_R), the relaxed emission is observed. In other words, under the conditions described above, excitation of either the F state or the R state yields emission spectra centered at λ_2. This situation is observed for a fluorophore in bulk, non-viscous solvents or at high temperatures. On the other hand, when the fluorophore is placed in a viscous medium or condensed phase or is frozen to very low temperatures, the relaxation time, which is a function of the rate of the physical reorientation of the solvent molecules, is drastically increased and is much longer than the fluorescence lifetime ($\tau_F << \tau_S$). Under these conditions, the blue shifted emission of the F state (emission maximum at λ_1) is observed with central excitation (λ_C) However, if the same system is now excited with lower energy quanta (λ_R) it will select a subclass of the total fluorophore population around which the solvent dipoles are oriented so as to decrease the energy difference between the ground and the excited states. In particular, this photoselected ground state is higher in energy and the photoselected excited state is at a lower energy level (see Fig. 1) because of the alignment of the solvent dipoles in the 'solvent-relaxed' orientation. Therefore, red edge excitation under these conditions selects those molecules which have a solvent-relaxed environment and resultantly, red shifted absorption and emission spectra (i.e., the emission spectrum is centered at λ_2 under these conditions). The third possibility is the situation where the rate of solvent reorientation is comparable to the fluorescence lifetime ($\tau_F \approx \tau_S$) i.e., under conditions of intermediate viscosity or temperature. In this case, excitation at λ_C gives rise to a spectrum centered at a wavelength intermediate between λ_1 and λ_2. This can be attributed to the fact that although the F state alone is initially excited, the comparable values of τ_F and τ_S give rise to emissions from both F and R states. This inhomogeneous spectrum thus also has a wider spectral distribution[27-29]. Upon red edge excitation, the emission occurs mainly from the R state, giving rise to a more red shifted emission spectrum which is also comparatively narrow, as it is composed of emission predominantly from the R state[17,19].

4 The Continuous Model

This model was first proposed by Bakhshiev and co-workers[30-32] to provide a phenomenological description of time-dependent effects of solvent-fluorophore interactions on emission spectra in terms of the observed spectral parameters (see Fig. 2). According to this model, the maximum of fluorescence emission [or more rigorously, the center of gravity of the emission spectrum, $\nu_m(t)$] is assumed to shift to lower energy in an exponential manner following excitation, with a characteristic relaxation time τ_S, *i.e.,*

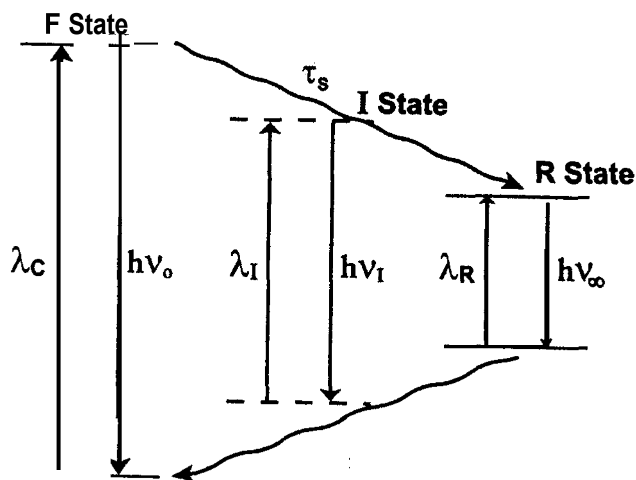

Fig. 2 The continuous model of solvent relaxation. The I state refers to one of the intermediate states between the initial (F) and the final (R) states, in which the solvent molecules are partially relaxed. ν_o, ν_I and ν represent the frequencies corresponding to the initially excited (Franck-Condon), intermediate, and completely relaxed states, respectively, while λ_C, λ_I, and λ_R denote the wavelength maxima associated with these states. τ_S is the reorientation time of the solvent molecules. See text (Section 4) for other details.

$$\nu_m\ (t) = \nu + (\nu_o - \nu)\ \exp\ (-\ t\ /\tau_S) \qquad \ldots (1)$$

where ν_o and ν represent the emission maxima (in cm^{-1}) of the initially excited (Franck-Condon) and the completely relaxed states. The spectral shape of the emission is assumed to remain constant during the time course of the emission. Due to the exponential nature of eq. (1), the emission maxima shift continuously from $\nu = \nu_o$ at $t = 0$ to $\nu = \nu_\infty$ at $t = \infty$. Therefore at $t = \tau_S$ In 2, the spectral shift is 50% complete.

However, in reality, there exists a statistical distribution of solvation states for an ensemble of polar fluorophores in solution, based on their dipolar interactions with the solvent molecules in both the ground and excited states. The molecules interacting in solution may differ in their mutual orientation and interaction energies which results in alteration of the energies of electronic transitions. Both the character of the energy distribution at the moment of excitation and its change with time (relaxation) will determine the spectroscopic behaviour of the system. Such a steady state spectrum will therefore be quite broad due to contributions from the various partially relaxed substates. This has been referred to as the 'dipole-orientational broadening' of the spectrum[17,20,22,33]. Taking all these into account, i.e., the statistical distribution of interaction energies for molecules with their environments, and photoselection of excited species according to the energy of the absorbed or emitted quanta, Demchenko later proposed a more realistic model[17,20,22].

5 The Wavelength-Selective Fluorescence Approach

In addition to the dependence of fluorescence emission maxima on the excitation wavelength (REES), fluorescence polarization and lifetime are also known to depend on the excitation and emission wavelengths in viscous solutions and in otherwise motionally restricted media. Taken together, these constitute the wavelength-selective fluorescence approach which consists 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 fluorophore in a complex biological system[8-10]. Early applications of REES and wavelength-selective fluorescence to systems of biological relevance have been restricted mainly to indole, tryptophan, and other fluorescent probes in viscous solvents[21].

6 The Unique Role of Tryptophan Residues in Membrane Proteins and Peptides: Tryptophan and the Membrane Interface

The presence of tryptophan residues as intrinsic fluorophores in most peptides and proteins makes them an obvious choice for fluorescence spectroscopic analyses of such systems. The role of tryptophan residues in the structure and function of membrane proteins has recently attracted a lot of attention[34,35]. The biological membrane provides a unique environment to membrane-spanning proteins and peptides thus influencing their structure and function. Membrane-spanning proteins have distinct stretches of hydrophobic amino acids that form the membrane-spanning domain and have been reported to have a significantly higher tryptophan content than soluble proteins[36]. In addition, it is becoming increasingly evident that tryptophan residues in integral membrane proteins and peptides are not uniformly distributed and that they tend to be localized toward the membrane interface, possibly because they are

involved in hydrogen bonding[37] with the lipid carbonyl groups or interfacial water molecules (see Fig. 3). For instance, crystal structures of membrane proteins such as the potassium channel[38] bacteriorhodopsin[39], maltoporin[40] and others have shown that most tryptophans are located in a saddle-like 'aromatic belt' around the membrane interfacial region. Statistical studies of sequence databases and available crystal structures of integral membrane proteins also show preferential clustering of tryptophan residues at the membrane interface[34,41,42]. Furthermore, for synthetic transmembrane peptides, tryptophan has been found to be an efficient anchor at the membrane interface[43] and defines the hydrophobic length of transmembrane helices[44]. Interestingly, the role of tryptophan residues in maintaining the structure and function of membrane proteins[34] has attracted considerable attention. The importance of tryptophan residues in maintaining the function of membrane proteins is exemplified by the fact that substitution or deletion of tryptophans often results in reduction or loss of protein functionality[45,46].

The preferential location of tryptophan residues at the membrane interface is thought to be due to the aromaticity of the indole moiety and the overall amphipathic nature of tryptophan[47]. The tryptophan rich aromatic belt at the membrane interface in transmembrane helices are thought to stabilize the helix with respect to the membrane environment[41]. The tryptophan residue has a large indole side chain that consists of two fused aromatic rings. In molecular terms, tryptophan is a unique amino acid since it is capable of both hydrophobic and polar interactions. In fact, the hydrophobicity of tryptophan. measured by partitioning into bulk solvents, has previously been shown to be dependent on the scale chosen[48]. Tryptophan ranks as one of the most hydrophobic amino acids on the basis of its partitioning into polar solvents such as octanol[49] while scales based on partitioning into nonpolar solvents like cyclohexane[50] rank it as only intermediate in hydrophobicity. This ambiguity results from the fact that while tryptophan has the polar -NH group which is capable of forming hydrogen bonds, it also has the largest nonpolar

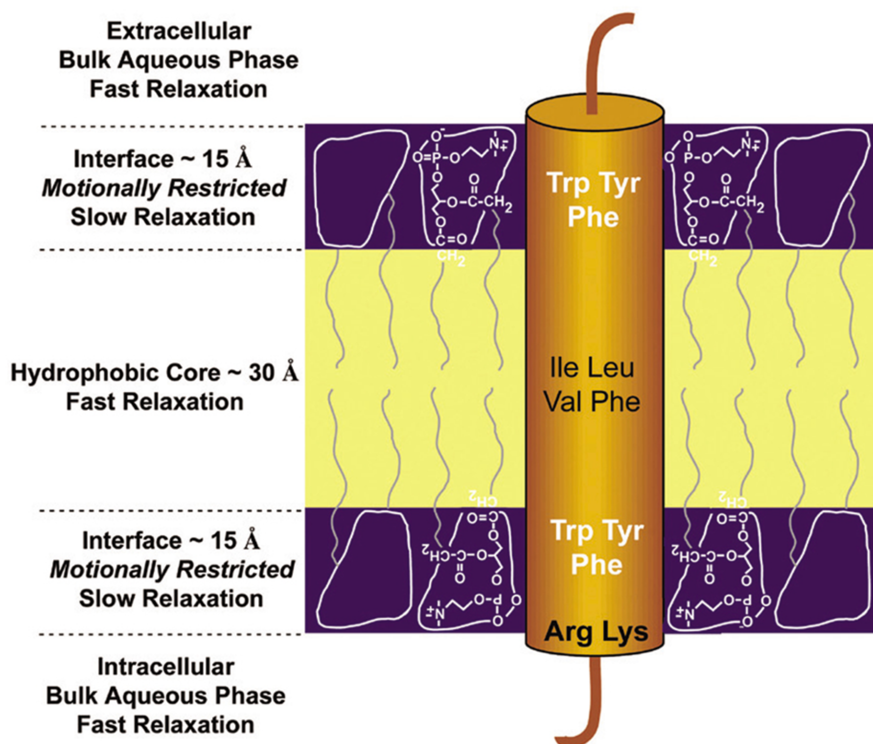

Fig. 3 A schematic representation of the membrane bilayer showing the various regions of the bilayer displaying motional anisotropy. The membrane lipids shown have two hydrophobic tails with a phosphatidylcholine (PC) headgroup. The preferred locations of various amino acids present in a membrane-spanning transmembrane domain are also shown. It is worth noting that the fluorescent tryptophan residues are localized in the membrane interface region, a region characterized by unique organization, dynamics, hydration and functionality. See text (Section 6) for other details. Adapted and modified from refs. [10] and [34].

accessible surface area among the naturally occuring amino acid[51]. Wimley and White have recently shown from partitioning of model peptides to membrane interfaces that the experimentally determined interfacial hydrophobicity of tryptophan is highest among the naturally occurring amino acid residues thus accounting for its specific interfacial localization in membrane-bound peptides and proteins[52]. Due to its aromaticity, the tryptophan residue is capable of π-π interactions and of weakly polar interactions[53]. The amphipathic character of tryptophan gives rise to its hydrogen bonding ability which could account for its orientation in membrane proteins and its function through long-range electrostatic interaction[46]. The amphipathic character of tryptophan also explains its interfacial localization in membranes due to its tendency to be solubilized in this region of the membrane, besides favourable electrostatic interactions and hydrogen bonding.

A direct consequence of highly organized molecular assemblies such as membranes is the restriction imposed on the mobility of molecules incorporated in such assemblies. It is well known that interiors of biological membranes are viscous, with the effective viscosity comparable to that of light oil[54,55]. The biological membrane, with its viscous interior, and distinct motional gradient along its vertical axis, thus provides an ideal system for the utilization of REES in particular and wavelength-selective fluorescence in general to study organization and dynamics of membrane-bound proteins and peptides. The use of this approach becomes all the more relevant in view of the fact that no detailed crystallographic database for membrane-bound proteins and peptides exists to date due to the inherent difficulty in crystallizing such molecules. Moreover, any information obtained has a dynamic component generally absent in X-ray crystallographic data.

The membrane exhibits a considerable degree of anisotropy along the axis perpendicular to the membrane plane. Properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds and extent of water penetration vary in a depth-dependent manner in the membrane. The interfacial region in membranes is therefore characterized by unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane (Fig. 3)[56-63]. It is a chemically heterogeneous region composed of lipid headgroups, water and portions of the acyl chain[64]. Overall, the interfacial region of the membrane accounts for 50% of the thermal thickness of the bilayer[60]. This specific region of the membrane exhibits slow rates of solvent relaxation and is also known to participate in intermolecular charge interactions[65] and hydrogen bonding through the polar headgroup[66-68]. These structural features which slow down the rate of solvent reorientation have previously been recognized as typical features of solvents giving rise to significant red edge effectsi6. It is therefore the membrane interface which is most likely to display red edge effects and is sensitive to wavelength-selective fluorescence measurements[69]. This makes membrane peptides and proteins ideally suited for studies using the wavelength-selective fluorescence approach since as discussed above, the fluorescent tryptophan residues are localized in this region of the membrane.

7 Application of the Wavelength-Selective Fluorescence Approach to Membrane Peptides

The membrane environment and the relative position within the membrane have a profound influence on the dynamics of amino acid residues of membrane spanning helices. Both natural and synthetic membrane peptides offer a convenient tool to study the specific role of membrane on the orientation, incorporation, stability and function of such peptides[70-73].

Tryptophan octyl ester (TOE) has been recognized as an important model for membrane-bound tryptophan residues[35,74-76]. The fluorescence characteristics of TOE incorporated into model membranes and membrane-mimetic systems have been shown to be similar to that of membrane-bound tryptophans. Consistent with the preferred interfacial location of the tryptophan residue and the solvent restricted environment of the interface, TOE has been found to exhibit REES. Further, the extent of REES was found to be dependent on pH indicating more motional freedom on deprotonation at higher pH[35].

Melittin, the major toxic component in the venom of the European honey bee, *Apis mellifera,* was one of the earlier membrane. peptides studied using the wavelength-selective fluorescence approach. Melittin is an amphipathic cationic hemolytic peptide with a single functionally

important tryptophan residue". Amphipathicity is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins[77,78]. This has made melittin very popular as a simple model to study lipid-protein interactions in membranes[77,79]. Results from wavelength-selective fluorescence studies showed that when bound to zwitterionic membranes, the micro-environment of the sole functionally active tryptophan of melittin was motionally restricted as evident from REES and other results in agreement with the interfacial location of the tryptophan residue[80]. Interestingly, wavelength-selective fluorescence studies indicate that the microenvironment of the tryptophan residue in melittin is modulated when bound to negatively charged membranes, and this could be related to the functional difference in the lytic activity of the peptide observed in the two cases[81]. REES is therefore sensitive to changes in dynamics of hydration caused by varying electrostatic interactions. In addition, REES of melittin bound to membranes (or membrane-mimetic systems) was found to be dependent on the lipid composition of the membrane and the hydration state of melittin[82,83].

In yet another study, the phenomenon of REES, -in conjunction. with time-resolved fluorescence spectroscopic parameters such as wavelength-dependent fluorescence lifetimes and time-resolved emission spectra (TRES) was utilized to study the localization and dynamics of the functionally important tryptophan residues in the gramicidin channel[84]. Gramicidin belongs to a family of prototypical channel formers which are naturally fluorescent due to the presence of four tryptophan residues[85]. These interfacially localized tryptopbans are known to play a crucial role in the organization and function of the channe1[45,46]. The results from the above study point out the motional restriction experienced by the tryptophans at the peptide-lipid interface of the gramicidin channel. This is consistent with other studies[45,46] in which such restrictions are thought to be imposed due to hydrogen bonding between the indole rings of the tryptophan residues in the channel conformation and the neighboring lipid carbonyls. The significance of such organization in terms of functioning of the channel is brought out by the fact that substitution, photodamage, or chemical modification of these tryptophans are known to give rise to channels with altered conformation and reduced conductivity[45,46,86-88]. More importantly, REES and related fluorescence approaches could be used to distinguish between the channel and non-channel conformation of gramicidin in membranes[89,90]. REES of gramicidin is therefore sensitive to the conformation adopted by the peptide in the membrane. This provides a convenient spectroscopic handle to monitor functional status of this important ion channel peptide. Further, REES of gramicidin incorporated into membranes was found to be dependent on the lipid composition[91] and phase state of the membrane[92].

Wavelength-selective fluorescence approach has also been used to monitor the membrane interaction of synthetic peptides corresponding to the functionally important regions of membrane binding proteins[93,94]. The 579-601 fragment of the ectodomain of the HIV- 1 gp41 protein which is essential for its activity, was shown to incorporate in model membranes[93]. Upon red edge excitation, there was a substantial red shift in fluorescence indicating a motionally restricted environment for the single tryptophan of this peptide. The REES result is somewhat unusual not only due to the rather large magnitude of REES (18 nm) but also due to the observation of REES for a peptide with the emission maximum at 348 nm when excited at the absorption maximum. This is in contrast with the previously accepted notion that REES can only be found for Trp residue emitting between 325 and 341 nm[95]. Large magnitude of REES (28 nm) has also been observed for the reporter tryptophan in membrane-bound PT-(1-46)F4W synthetic peptide which corresponds to the o-loop region of the human prothrombin γ-carboxyglutamic acid domain[94]. In both these cases, the peptides were found to partition to the inter-facial region of the membrane. The observed REES, would therefore be a direct consequence of the motionally restricted membrane interface%. A large REES (25 nm) is also displayed by the NBD-labelled peptide fragment of apolipoprotein C-II (apoC-II$_{19\text{-}39}$) when bound to egg yolk phosphatidylcholine membranes[97]. This observation, along with results from time-resolved fluorescence measurements, points out that the NBD-labelled peptide is located near the polar-inter-facial region of the bilayer where it experiences a heterogeneous environment.

The interaction of the peptide corresponding to the γM4 transmembrane domain of the nicotinic acetylcholine receptor with membranes has recently been investigated[98]. Wavelength-selective fluorescence showed that the helix is oriented in such a way that the N-terminal tryptophan (Trp 453) is located in a motionally restricted environment at the membrane interface, confirming the previous observations that γM4 forms a transmembrane helix[99]. The topology and bilayer location of tryptophan residues in the colicin El channel peptide has very recently been explored using a rather novel approach in which REES of single tryptophan containing channel peptides was determined in the soluble aqueous state and when bound to membranes[100]. From the difference in REES values of the channel peptides in the membrane-bound and soluble state, the topology of the peptide was mapped out.

8 Application of the Wavelength-Selective Fluorescence Approach to Membrane Proteins

An early application of the wavelength-selective fluorescence approach to study membrane protein organization consisted of studying the microconformational heterogeneity of the membrane binding domain of cytochrome b_5 by comparing the information obtained from the native protein and its mutant which has a single tryptophan residue in this domain[101]. Both these proteins show a red shift in the emission spectrum when excited at the long wavelength edge of the excitation spectrum indicating thereby that the tryptophan residue(s) in both cases are localized in a region of motional constraint.

In a later study, the tryptophans of the pore-forming *Staphylococcus aureus* α-toxin were shown to exhibit REES[102]. However, no significant difference was observed between REES displayed by the soluble and the membrane-bound forms of the toxin indicating no drastic change in the tryptophan environment upon membrane binding. The tryptophans in the *E. coli* porin Omp-F, a pore forming channel protein, also exhibits REES[103].

Membrane active proteins that exist in soluble and membrane-bound forms undergo considerable conformational change in the transition from the soluble to the membrane-bound state. One such protein is the mitochondrial creatine kinase which shows a large increase in REES upon membrane binding[104]. The soluble form exhibits REES of 6 nm which increases to a REES of 19 nm in the presence of membranes which is indicative of a restricted environment for the tryptophans in the membrane-bound form. Since the tryptophan residues are localized in the protein interior, the observed motional restriction has been attributed to conformational change of the protein induced by lipid binding.

9 Conclusions and Future Perspectives

Water plays a crucial role in the formation and maintenance of cellular architecture. Knowledge of dynamics of hydration at the molecular level is thus of considerable importance in understanding cellular structure and function[105-112]. As mentioned earlier, REES 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 biological systems, the ubiquitous solvent is water, the information obtained in such cases will come from the otherwise 'optically silent' water molecules. The unique feature about REES is that while all other fluorescence techniques such as fluorescence quenching, energy transfer and polarization measurements yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics which is not possible to obtain by other techniques. This makes the use of REES and the wavelength-selective fluorescence approach powerful in membrane biology since hydration plays a crucial modulatory role in a large number of cellular events involving the membrane such as lipid-protein interactions[107] and ion transport[105-107]. REES and the wavelength-selective fluorescence approach, in combination with the novel molecular biology approaches for site-specific incorporation of unnatural fluorophores at specific sites[113], could prove to be an extremely powerful tool to probe organization and dynamics of membrane proteins and peptides.

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References

1 J Deisenhofer, O Epp, K Miki, R Huber and H Michel *Nature* **318** (1985) 618

2 J U Bowie *Curr Opin Struct Biol* **11** (2001) 397

3 W L Smith, R M Garavito and S Ferguson-Miller J *Biol Chem* **276** (2001) 32393

4 S J Opella *Nat Struct Biol* **4** (NMR Suppl.) (1997) 845

5 J R Lakowicz J *Biochem Biophys Meth* **2** (1980) 91

6 A Chattopadhyay *Biomembrane Structure and Function: The State of theArt* (Eds. B P Gaber and K R K Easwaran) Adenine Press Schenectady New York (1992) 153

7 A P Demchenko *Topics in Fluorescence Spectroscopy* (Vol. 3) (Ed. J R Lakowicz) Plenum Press New York (1992) 65

8 S Mukherjee and A Chattopadhyay *J Fluorescence* **5** (1995) 237

9 A Chattopadhyay *Fluorescence Spectroscopy, Imaging and Probes* (Eds. R Kraayenhof, A J W G Visser, H C Gerritsen and O Wolfbeis) Springer-Verlag Heidelberg (2002) 211

10 A Chattopadhyay *Chem Phys Lipids* **122** (2002) 3

11 J B Birks *Photophysics of Aromatic Molecules* Wiley-Interscience London (1970)

12 K K Rohatgi-Mukherjee *Fundamentals of Photochemistry* Wiley Eastern New Delhi (1978)

13 R F Chen *Anal Biochem* **19** (1967) 374

14 A N Fletcher *J Phys Chem* **72** (1968) 2742

15 W C Galley and R M Purkey *Proc Natl Acad Sci USA* **67** (1970) 1116

16 K I Itoh and T Azumi. *J Chem Phys* **62** (1975) 3431

17 A P Demchenko *Biophys Chem* **15** (1982) 101

18 J R Lakowicz and S Keating-Nakamoto *Biochemistry* **23** (1984) 3013

19 R B Macgregor and G Weber *Ann N Y Acad Sci* **366** (1981) 140

20 A P Demchenko *Ultraviolet Spectroscopy of Proteins* Springer-Verlag Heidelberg (1986)

21 A P Demchenko *Trends Biochem Sci* **13** (1988) 374

22 A P Demchenko and A S Ladokhin *Eur Biophys J* **15** (1988) 369

23 S Mukherjee, A Chattopadhyay, A Samanta and T Soujanya *J Phys Chem* **98** (1994) 2809

24 A P Demchenko *Luminescence* **17** (2002) 19

25 J R Lakowicz and A Balter *Biophys Chem* **15** (1982) 353

26 J R Lakowicz and A Balter *Photochem Photobiol* **36** (1982) 125

27 J R Lakowicz *Principles of Fluorescence Spectmscopy* Kluwer-Plenum New York (1999)

28 G Weber and F J Farris *Biochemistry* **18** (1979) 3075

29 J R Lakowicz, H Cherek, G Laczko and E Gratton *Biochim Biophys Acta 777* (1984) 183

30 N G Bakhshiev, Yu T Mazurenko and I V Piterskaya *Opt Spectrosk* **21** (1966) 307

31 Yu T Mazurenko and N G Bakhshiev *Opt Spectrosk* **28** (1970) 490

32 N G Bakhshiev, Yu T Mazurenko and IV Piterskaya *Izv Akad Nauk SSSR Ser Fiz* **32** (1969) 1262

33 A P Demchenko and A I Sytnik *J Phys Chem* **95** (1991) 10518

34 R A F Reithmeier *Curr Opin Struct Biol* **5** (1995) 491

35 A Chattopadhyay, S Mukherjee, R Rukmini, S S Rawat and *S* Sudha *Biophys J* **73** (1997) 839

36 M Schiffer, C H Chang and F J Stevens *Protein Engineering* **5** (1992) 213

37 J A Ippolito, R S Alexander and D W Christianson *J Mol Biol* **215** (1990) 457

38 D A Doyle, J Morais Cabral, R A Pfuetzner, A Kuo, J M Gulbis, S L Cohen, B T Chait and R MacKinnon *Science* **280** (1998) 69

39 H Luecke, B Schobert, H-T Richter, J-P Cartailler and J K Lanyi *Science* **286** (1999) 255

40 T Schirmer, T A Keller, Y F Wang and J P Rosenbusch *Science* **267** (1995) 512

41 C Landolt-Marticorena, K A Williams, C M Deber and R A F Reithmeier *J Mol Biol* **229** (1993) 602

42 M B Ulmschneider and M S P Sansom *Biochim Biophys Acta* **1512** (2001) 1

43 M R R de Planque, D V Greathouse, R E Koeppe, H Schafer, D Marsh and J A Killian *Biochemistry* **37** (1998) 9333

44 J AA Demmers, E van Duijn, J Haverkamp, D V Greathouse, R E Koeppe, A J R Heck and J A Killian *J Biol Chem* **276** (2001) 34501

45 M D Becker, DV Greathouse, R E Koeppe and O S Andersen *Biochemistry* **30** (1991) 8830

46 V Fonseca, P Daumas, L Ranjalahy-Rasoloarijao, F Heitz, R Lazaro, Y Trudelle and O S Andersen *Biochemistry* **31** (1992) 5340

47 W-M Yau, W C Wimley, K Gawrisch and S H White *Biochemistry* **37** (1998) 14713

48 J L Fauchere *Trends Biochem Sci* **10** (1985) 268

49 J L Fauchere and V Pliska *Eur J Med Chem* **18** (1983) 369

50 A Radzicka and R Wolfenden *Biochemistry* **27** (1988) 1664

51 W C Wimley and S H White *Biochemistry* **31** (1992) 12813

52 W C Wimley and S H *White Nat Struct Biol* **3** (1996) 842

53 S K Burley and G A Petsko *Adv Protein Chem* **39** (1988) 125

54 R A Cone *Nature New Biol* **236** (1970) 39

55 M M Poo and R A Cone *Nature* **247** (1972) 438

56 J Seelig *Quart Rev Biophys* **10** (1977) 353

57 R G Ashcroft, H G L Coster and J R Smith *Biochim Biophys Acta* **643** (1981) 191

58 C D Stubbs, S R Meech, A G Lee and D Phillips *Biochim Biophys Acta* **815** (1985) 351

59 E Perochon, A Lopez and J F Tocanne *Biochemistry* **31** (1992) 7672

60 S H White and W C Wimley Curr *Opin Struct Biol* **4** (1994) 79

61 S J Slater, C Ho, F J Taddeo, M B Kelly and C D Stubbs *Biochemistry* **32** (1993) 3714

62 R M Venable, Y Zhang, B J Hardy and R W Pastor *Science* **262** (1993) 223

63 K Gawrisch, J A Barry, L L Holte, T Sinnwell, L D Bergelson and J A Ferretti *Mol Memb Biol* **12** (1995) 83

64 M C Wiener and S H White *Biophys J* **61** (1992) 434

65 P Yeagle *The Membranes of Cells* Academic Press Orlando, Florida (1987) p 89

66 R B Gennis *Biomembranes: Molecular Structure and Function* Springer-Verlag New York (1989) p 47

67 T B Shin, R Leventis and J R Silvius *Biochemistry* **30** (1991) 7491

68 J M Boggs *Biochim Biophys Acta* **906** (1987) 353

69 A Chattopadhyay and S Mukherjee *Biochemistry* **32** (1993) 3804

70 L A Chung *Biomembrane Structure and Function: The State of the Art* (Eds. B P Gaber and K R K Easwaran) Adenine Press Schenectady New York (1992) 133

71 J A Lundbaek, A M Maer and O S Andersen *Biochemistry* **36** (1997) 5695

72 R J Webb, J M East, R P Sharma and A G Lee *Biochemistry* **37** (1998) 673

73 J Ren, S Lew, J Wang and E London *Biochemistry* **38** (1999) 5905

74 A S Ladokhin and P W Holloway *Biophys J* **69** (1995) 506

75 B de Foresta, J Gallay, J Sopkova, P Champeil and M Vincent *Biophys J* **77** (1999) 3071

76 B Sengupta and P K Sengupta *B&hem Biophys Res Commun* **277** (2000) 13

77 C E Dempsey *Biochim Biophys Acta* **1031** (1990) 143

78 Y Shai *Trends Biochem Sci* **20** (1995) 460

79 G Saberwal and R Nagaraj *Biochim Biophys Acta* **1197** (1994) 109

80 A Chattopadhyay and R Rukmini *FEBS Lett* **335** (1993) 341

81 A K Ghosh, R Rukmini and A Chattopadhyay *Biochemistry* **36** (1997) 14291

82 H Raghuraman and A Chattopadhyay (unpublished observations)

83 H Raghuraman and A Chattopadhyay (unpublished observations)

84 S Mukherjee and A Chattopadhyay *Biochemistry* **33** (1994) 5089

85 R E Koeppe and O S Andersen *Annu Rev Biophys Biomol Struct* **25** (1996) 231

86 F Heitz, G Spach and Y Trudelle *Biophys J* **40** (1982) 87

87 D Jones, E Hayon and D Busath *Biochim Biophys Acta* **861** (1986) 62

88 M Strassle, G Stark, M Wilhelm, P Daumas, F Heitz and R Lazaro *Biochim Biophys Acta* **980** (1989) 305

89 S S Rawat *Lipid-Protein Interaction: Organization and Dynamics of the Ion Channel-Forming Peptide Gramicidin in Membranes and Micelles* Ph.D. thesis, Jawaharlal Nehru University, New Delhi, India (1998)

90 S S Rawat, D A Kelkar and A Chattopadhyay (unpublished observations)

91 S S Rawat and A Chattopadhyay *Prog Biophys Mol Biol* **65** (Suppl 1) (1996) 110

92 D A Kelkar and A Chattopadhyay (unpublished observations)

93 N C Santos, M Prieto and M A R B Castanho *Biochemistry* **37** (1998) 8674

94 L A Falls, B C Furie, M Jacobs, B Furie and A C Rigby *J Biol Chem* **276** (2001) 23895

95 A P Demchenko *Eur Biophys J* **16** (1988) 121

96 A Chattopadhyay and S Mukherjee *Langmuir* **15** (1999) 2142

97 C E MacPhee, G J Howlett, W H Sawyer and A H A Clayton *Biochemistry* **38** (1999) 10878

98 R F M de Almeida, L M S Loura, F J Barrantes and M Prieto *Biophys J* **80** (2001) 545a

99 F J Barrantes, S S Antollini, M P Blanton and M Prieto **J** *Biol Chem* **275** (2000) 37333

100 M C Tory and A R Merrill *Biochim Biophys Acta* **1564** (2002) 435

101 A S Ladokhin, L Wang, A W Steggles and P W Holloway *Biochemistry* **30** (1991) 10200

102 S M Raja, S S Rawat, A Chattopadhyay and A K Lala *Biophys J* **76** (1999) 1469

103 B R Patnaik, S Ghosh and M R Rajeswari *Biochem Mol Biol Intl* **42** (1997) 173

104 T Granjon, M-J Vacheron, C Vial and R Buchet *Biochemistry* **40** (2001) 6016

105 J H Crowe and L M Crowe *Biol Membr* **5** (1984) 57

106 R P Rand and V A Parsegian *Biochim Biophys Acta* **988** (1989) 351

107 C Ho and C D Stubbs *Biophys J* **63** (1992) 897

108 W B Fischer, S Sonar, T Marti, H G Khorana and K J Rothschild *Biochemistry* **33** (1994) 12757

109 H Kandori, Y Yamazaki, J Sasaki, R Needleman, J K Lanyi and A Maeda *J Am Chem Soc* **117** (1995) 2118

110 R Sankararamakrishnan and M S P Sansom *FEBS Lett* **377** (1995) 377

111 D Häussinger *Biochem J* **313** (1996) 697

112 P Mentré (Ed.) *Cell Mol Biol* **47** (2001) 709

113 B E Cohen, T B McAnaney, E S Park, Y N Jan, S G Boxer and L Y Jan *Science* **296** (2002) 1700