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Wavelength-Selective Fluorescence as a Novel Tool to Study Organization and Dynamics in Complex Biological Systems

Sushmita Mukherjee[1] and Amitabha Chattopadhyay[1,2]

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The dynamics exhibited by a given component of a large macromolecule such as a folded globular protein or an organized supramolecular assembly like the biological membrane is a function of its precise localization within the larger system. A set of approaches based on the red edge effect in fluorescence spectroscopy, which can be used to monitor *directly* the environment and dynamics around a fluorophore in a complex biological system, is reviewed in this article. 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 the absorption band, is termed the 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. This phenomenon arises from the slow rates of solvent relaxation 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 in biology since hydration plays a crucial modulatory role in a large number of important cellular events.

KEY WORDS: REES; solvent dipole reorientation; wavelength-dependent fluorescence polarization and lifetimes; proteins; membranes.

INTRODUCTION

In order to study the structure–function relationship in any biological macromolecule such as a peptide, protein, or nucleic acid or in an organized supramolecular assembly like the biological membrane or a protein–nucleic acid complex, it is necessary to appreciate the dynamics of the constituent molecules as well as that of the system as a whole. Although very detailed and precise structural information regarding some of these macromolecules can be obtained from crystallographic diffraction data, such information is necessarily static. Further, in the case of membrane-embedded proteins and peptides, a detailed crystallographic database is not available due to the inherent difficulty in crystallizing membrane-bound molecules. Spectroscopic techniques, which provide both structural as well as dynamic information, thus become extremely useful for analyses of such systems. Fluorescence spectroscopy offers one such approach that has found wide-ranging applications in a variety of systems of biological relevance. The advantages of using fluorescence techniques, in addition to the dynamic nature of the information obtained, are its sensitivity, suitable time scale, noninvasive nature, and minimal perturbation.[1-5]

The dynamics exhibited by a given component in a complex biological system is thus a function of its

[1] Centre for Cellular and Molecular Biology, Hyderabad 500 007, India.
[2] To whom correspondence should be addressed.

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precise localization within the larger system. For instance, the membrane is a highly anisotropic medium largely confined to two dimensions, whose hydrophobic interior cannot be mimicked by any isotropic organic solvent. While the center of the bilayer is nearly isotropic, the upper portion, only a few angstroms away toward the surface of the membrane, is highly ordered.[6–10] Not only does this result 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 location (depth) in the membrane. Similarly, in case of a folded globular protein, the environment experienced by a given amino acid residue or a fluorescence probe, either covalently attached or partitioned from the aqueous phase, will be largely dependent on whether it is buried in the hydrophobic interior of the protein or exposed to the external aqueous milieu.

Such environment-induced motional restriction experienced by a given fluorophore has been previously used to characterize the microenvironment of a given fluorophore. This motional restriction has been variously termed as 'fluidity' or 'microviscosity'.[11–14] These studies have utilized techniques such as fluorescence depolarization (which is a function of the rate of rotational diffusion of a fluorophore in the excited state) or excimer formation (which is a function of the lateral mobility of the fluorophore). In addition to fluorescence spectroscopy, 'order parameters' calculated from the electron spin resonance hyperfine splittings and deuterium nuclear magnetic resonance quadrupolar splittings have also been used to represent localized motional characteristics.[15,16]

However, all the above approaches share one major limitation. If the probe under study is freely mobile [e.g., in cases of 1,6-diphenyl-1,3,5-hexatriene (DPH) depolarization or pyrene excimer formation], it tends to exhibit a wide spatial distribution since it is not uniquely localized. Any information about microviscosity obtained from such studies is thus at best a representation averaged over the whole range of their spatial distribution. On the other hand, if the probe is localized by covalent attachment to a lipid or peptide, then this attachment itself may prove to be a major hindrance to the motional freedom of the probe molecule, thereby overestimating the environment-induced motional restriction imposed on it. In this review, we discuss a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to monitor *directly* the environment and dynamics around the fluorophore. The red edge effect arises from the slow rates (when compared to the fluorescence lifetime) of solvent relaxation around an excited-state fluorophore, which is a function of the environment-induced motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. This approach thus allows one to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) utilizing the fluorophore merely as a reporter group.

THE RED EDGE EFFECT

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.[17,18] It is obvious from this rule that fluorescence, in principle, should be independent of wavelength of excitation. In fact, such a lack of dependence on excitation wavelength is often taken as a criterion for purity and homogeneity of a molecule. Thus, for a fluorophore in a bulk nonviscous solvent, the fluorescence decay rates and the wavelength of maximum emission are usually independent of the excitation wavelength.

However, this generalization breaks down in the 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 below). 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).[19–26] Since REES is observed only under conditions of restricted mobility, it serves as an indicator of fluorophore environment.

The origin of the red edge effect 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.[21,24–30] For a polar fluorophore, a di-

polar interaction with the solvent molecules occurs in the ground state in order to minimize the energy of the given state. Since the dipole moment (magnitude as well as direction) of a molecule changes upon excitation, the solvent dipoles have to reorient around this new excited-state dipole moment of the fluorophore so as to attain an energetically favorable 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 in about the same time scale as the process of excitation of the fluorophore itself (10^{-15} s). 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 nonviscous solvent, this reorientation time τ_S is of the order of 10^{-12} s, 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} s.[3] Hence, irrespective of the excitation wavelength used, 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 is now of the order of 10^{-9} s or longer. Under these conditions, excitation at 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 a way similar to that found in the solvent-relaxed state. Thus, the necessary condition for REES is that different fluorophore populations are excited at the maximal and the red edge excitation and, more importantly, that this difference is maintained in the time scale of fluorescence lifetime. As discussed above, this requires that the dipolar relaxation time for the solvent shell be comparable to or longer than the fluorescence lifetime. 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 thus be summarized as follows: (1) The fluorophore should normally be polar so as to be able to orient suitably the neighboring solvent molecules in the ground state; (2) the solvent molecules surrounding the fluorophore should be polar; (3) the solvent reorientation time τ_S around the excited-state dipole moment should be comparable to or longer than the fluorescence lifetime τ_F; and (4) 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 on properties of the environment interacting with it (which is a function of τ_S). It has recently been shown for 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled phospholipids incorporated into model membranes that a dipole moment change of 3.5–3.9 D upon excitation is enough to give rise to significant red edge effects.[32]

THE TWO-STATE MODEL

The two-state model provides a simple conceptual framework within which one can consider the phenomenon of solvent relaxation.[26,33–35] 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 occurred. 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 the general and the 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 moment. Now, 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 three possibilities exist (see 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 emis-

[3] 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.[31] 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.

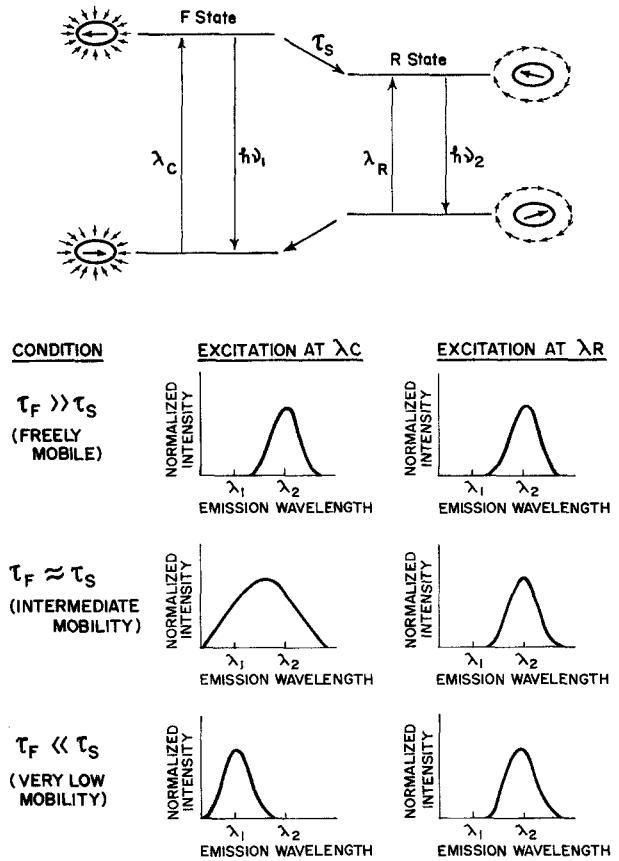

Fig. 1. A schematic representation of the two-state model of solvent relaxation in terms of energy levels (top) and spectra (bottom). F and R states refer to the Franck–Condon (or initial) and relaxed (or final) excited states, respectively. τ_S is the reorientation time of the solvent molecules and 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 with direct excitation of the Franck–Condon and relaxed states. See text for other details.

sion 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, nonviscous 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 now 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 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. Here, 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.[35–37] 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.[25,27]

THE CONTINUOUS MODEL

This model was first proposed by Bakhshiev and co-workers[38–40] 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, $_m(t)$] is assumed to shift to lower energy in an exponential fashion following excitation, with a characteristic relaxation time τ_S, *i.e.*,

$$\bar{\nu}_m(t) = \bar{\nu}_\infty + (\bar{\nu}_0 - \bar{\nu}_\infty)e^{-t/\tau S} \qquad (1)$$

where $\bar{\nu}_0$ and $\bar{\nu}_\infty$ represent the emission maxima (in cm^{-1}) of the initially excited (Franck–Condon) and the completely relaxed states, respectively. 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 $\bar{\nu} = \bar{\nu}_0$ at $t = 0$ to $\bar{\nu} = \bar{\nu}_\infty$ at $t = \infty$. Therefore at $t = \tau_S$ ln 2, the spectral shift is 50% complete.

However, in reality, there exists a statistical distribution of solvation states for an ensemble of polar fluo-

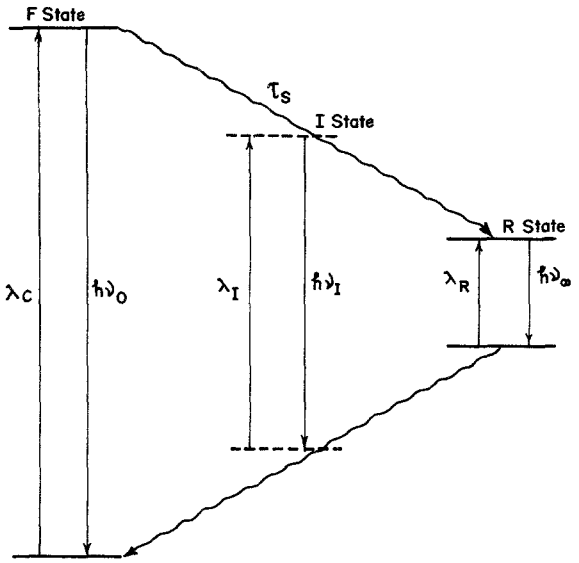

Fig. 2. The continuous model of solvent relaxation. The I state refers to one of the intermediate states between the initial (F) and the final (R) states, in which the solvent molecules are partially relaxed. ν_0, ν_I, and ν_∞ represent the frequencies corresponding to the initially excited (Franck–Condon), 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. See text for other details.

rophores 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 behavior 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.[25,28,30,41] 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.[25,28,30]

ELUCIDATION OF PROTEIN CONFORMATION

The dynamic properties of the protein matrix surrounding a given amino acid residue or a fluorophore either covalently attached to or partitioned from the aqueous phase in case of a globular protein can be examined from the rate at which this matrix responds to (or relaxes around) the newly created excited-state dipole moment of the fluorophore. In other words, the magnitude of REES could be utilized to estimate the relative rigidity of the region of the protein surrounding the fluorophore. Pioneering work in the application of this effect to proteins was carried out by Demchenko and co-workers.[25,28–31,42–45] In one of their early studies,[25] it was shown that the fluorescence emission spectra of 2-(*p*-toluidinylnaphthalene)-6-sulfonate (TNS) associated with proteins such as β-lactoglobulin, β-casein, and bovine and human serum albumins depend on the excitation wavelength used, giving rise to REES of the order of 10 nm in all cases. The fact that this effect was actually a result of the slow rate of solvent dipolar relaxation was confirmed when a similar effect was observed in case of the same fluorophore in glucose glass and in glycerol at 1°C, but not in liquid solutions.[25] Such red edge effects have also been observed with other proteins bound to TNS[26,42,46] or to 2'-(N,N-dimethyl)amino-6-naphthoyl-4-*trans*-cyclohexanoic acid (DANCA),[47] as well as by utilizing the intrinsic tryptophan fluorescence of proteins and peptides.[43,44,48,49] Interestingly, a thorough analysis of a variety of single-tryptophan-containing proteins in terms of their ability to exhibit REES yielded the following pattern.[43] It was found that REES was not observed both for proteins (such as azurin, a calcium form of whiting parvalbumin, and ribonucleases T_1 and C_2) having very short wavelength fluorescence emission maxima (between 307 and 323 nm), and for those (such as myelin basic protein, β-casein, monomeric form of melittin) with extremely long wavelength emission (between 341 and 350 nm). However, considerable REES effects were observed for many proteins (such as tetrameric form of melittin, human serum albumin in F form, albumin complexed with sodium dodecyl sulfate) with intermediate maxima of emission (between 325 and 341 nm). It was suggested that in case of proteins emitting at very short wavelengths, the tryptophan residues were in a hydrophobic environment and the dipole-orientational broadening of the spectra was insufficient to create a large enough distribution of differentially solvated substates that could be photoselected in order to give rise to REES. On the other hand, in case of the very long wavelength-emitting proteins, the tryptophans were exposed to an aqueous environment undergoing much faster solvent relaxation as compared to the fluorescence lifetimes of the concerned tryptophans. Therefore, only for the proteins that emit in the intermediate-wavelength range is there enough range of dipole-orientational broadening

and, at the same time, is the rate of solvent reorientation slow enough to give rise to this effect.

In addition to the application of REES to study protein conformation in solution, this phenomenon has also been applied to more complex systems, such as the intact eye lens. The eye lens in its normal state is a clear, transparent, and highly refractive cellular body. About 35% by weight of the lens is made up of three closely related proteins called crystallins whose organization and intermolecular interactions have been shown to play a crucial role in maintaining the transparency of the lens.[50,51] The magnitude of REES has been effectively utilized as a parameter to study the photophysical and chemical properties of isolated intact eye lenses and to probe the change in the organization of the lens upon photodamage.[52] The use of REES has very recently been further extended to relate the properties of the lens with those of its constituent proteins and their homo- and heteroaggregates.[53] The properties of intact eye lenses from different species have also been compared to study species-dependent variation of mobility and packing of the crystallins.[53]

ELUCIDATION OF MEMBRANE STRUCTURE: ORGANIZATION AND DYNAMICS

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.[6–10] Not only does this result 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[54,55] 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, 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.[56]

Few initial reports in which excitation wavelength dependence of emission maxima of the fluorescence probes TNS or ANS (1-anilinonaphthalene-8-sulfonate) in model lecithin membranes were investigated indicated no appreciable red shift.[26,57] However, REES was reported in case of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles labeled with 6-palmitoyl-2[[[2-(trimethylammonio)ethyl]amino]methyl]naphthalene chloride (Patman), an amphiphilic phase-sensitive probe.[58] The above results can be rationalized on the basis of the location of these probes in the membrane. On one hand, both TNS and ANS are charged at neutral pH and therefore access the phospholipid headgroup from the external aqueous phase. This implies that for these probes the immediate environment will be the aqueous phase adjacent to the headgroup, where solvent relaxation is extremely fast, and, as such, no red edge effect can be expected. On the other hand, because of the fatty acyl chain in Patman, it partitions well into the membrane and therefore experiences a much more motionally restricted environment.

The choice of a suitable probe is thus of utmost importance in designing membrane-active molecules capable of exhibiting REES. It is desirable that the probe be polar and be able to strongly partition into the membrane and intercalate with its normal components, i.e., the phospholipids. Further, the fluorescent portion of the molecule should be suitably embedded in the membrane. REES is indeed observed when the above criteria are satisfied.[58–63] In addition, it is preferable that the membrane-embedded molecule has only one fluorescent group and that it has a unique location in the membrane and not a distribution of locations. Such probes can be used to correlate the extent of REES with a specific fluorophore environment, which in case of such uniquely localized probes translates to a specific region of the membrane. One such probe that has been employed to study the phenomenon of REES and related effects in membranes is the widely used lipid probe NBD-PE [N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine].[59,60] In this probe, the fluorescent NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule. This study is significant in regard to membrane organization, because the precise orientation and location of the NBD group of this molecule in the membrane are known.[64–70] This group has been found to be localized at the membrane interface, which has unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane.[6–8,71–73] In another study, the red edge effect and other related approaches have been utilized to probe the

environment of the sole tryptophan residue of membrane-bound melittin, the principal toxic component in the venom of the European honey bee, *Apis mellifera*.[61] These studies revealed that the tryptophan residue of melittin is localized in a motionally restricted region of the membrane, probably at the membrane interface.

The phenomenon of REES, in conjunction with time-resolved fluorescence spectroscopic parameters such as wavelength-dependent fluorescence lifetimes (see below) and time-resolved emission spectra (TRES), has been further utilized to study the localization and dynamics of the functionally important tryptophan residues in the gramicidin channel.[62] Gramicidin belongs to a family of prototypical channel formers which are naturally fluorescent due to the presence of tryptophan residues. These tryptophans are known to play a crucial role in the organization and function of the channel.[74–77] The results from the above study[62] point out the motional restriction experienced by the tryptophans at the peptide–lipid interface of the gramicidin channel. This is consistent with other studies in which such restrictions are thought to be imposed due to hydrogen bonding between the indole rings of the tryptophans and the neighboring lipid carbonyls.[74,76] Furthermore, time-resolved fluorescence studies indicate a heterogeneous environment for these tryptophans, which can be grouped into at least two classes experiencing very different microenvironments. These results also indicate the presence of aromatic–aromatic interactions between two of the tryptophan residues in the channel conformation. The significance of such organization in terms of the functioning of the channel is brought out by the fact that substitution, photodamage, or chemical modification of these tryptophans gives rise to channels with altered conformation and reduced conductivity.[74–77]

FLUORESCENCE LIFETIMES: EFFECT OF EXCITATION AND EMISSION WAVELENGTHS

The origin of the red edge effect lies in differential extents of solvent reorientation around the excited-state fluorophore, with each excitation wavelength selectively exciting a different average population of fluorophores. Since fluorescence lifetimes are known to be sensitive to excited-state interactions, differential extents of solvent relaxation around a given fluorophore could be expected to give rise to differences in its lifetime. Under conditions where red edge effects are observed, a marked shortening is found in the mean lifetimes of the fluorophores.[23,42,60–62,78,79] Such a decrease in the mean lifetime of a fluorophore with increasing excitation wavelength in an environment of restricted mobility could be rationalized as follows. When such a fluorophore is excited at the mean excitation wavelength, the majority of the fluorophores will emit at the steady-state fluorescence emission maximum (which is also the monitoring wavelength for the acquisition of fluorescence intensity decays), and will have a certain mean lifetime associated with them. However, if the same fluorophore is now excited at the red edge of the absorption band without altering the emission wavelength at which the fluorescence decay is being monitored (which is now no longer the emission maximum), the lifetime will be shortened because of the preselection of only those fluorophores which have emitted early, and hence the solvent reorientation has not been allowed to shift the emission wavelength to the average (steady-state) maximum of fluorescence emission for that excitation wavelength.

On the other hand, when a polar fluorophore is placed in an environment of restricted mobility, its mean lifetime is found to increase steadily with increasing emission wavelength when the excitation wavelength is kept constant.[57,58,60–62,80–86] Such increasing lifetimes across the emission spectrum may be interpreted in terms of solvent reorientation around the excited-state fluorophore as follows. Observation at shorter wavelengths of emission selects unrelaxed fluorophores. Their lifetime is shorter because this population is decaying, both at the rate of fluorescence emission at the given excitation wavelength and by decay to longer (unobserved) wavelengths. In contrast, observation at the long-wavelength (red) edge of the emission selects the relaxed fluorophores, which have spent enough time in the excited state to allow increasingly larger extents of solvent reorientation.

FLUORESCENCE POLARIZATION: EFFECT OF EXCITATION AND EMISSION WAVELENGTHS

In addition to the dependence of fluorescence emission maxima and lifetimes on the excitation wavelength, fluorescence polarization is also known to depend on the excitation wavelength in viscous solutions or in otherwise motionally restricted media.[28,35,60–62,79,87–94] Valeur and Weber[79,92] observed an increase in polarization with increasing excitation wavelength for aromatic fluorophores such as naphthylamines. These results were interpreted as indicating a larger apparent molecular volume, which in turn was explained as the result of an out-of-plane electronic transition, which requires more displacement of solvent than an in-plane transition. They

suggested that these out-of-plane transitions are selectively excited upon red edge excitation. Weber and Shinitzky[90] had previously suggested that such an effect could be interpreted as the failure of self-energy transfer. Lakowicz[93] proposed an alternate explanation for the same observation. According to this proposition, there is a decreased rotational rate of the fluorophore in the relaxed state, because of strong dipolar interactions with the surrounding solvent molecules. On red edge excitation, a selective excitation of this subclass of fluorophores occurs. Because of strong interactions with the polar solvent molecules in the excited state, one may expect these 'solvent-relaxed' fluorophores to rotate more slowly, thereby increasing the polarization.

Fluorescence polarization is also found to be dependent on the emission wavelength in case of a motionally restricted fluorophore. Under such conditions, considerable decrease in polarization is observed with increasing emission wavelength.[57,58,60–62,85,95] This observation can be rationalized as follows. As has been discussed above, the mean lifetime of a fluorophore increases with increasing emission wavelength. Such longer-lived fluorophores, which are also those that emit at higher wavelengths, therefore have more time to rotate in the excited state, giving rise to lower polarization.

CONCLUSION AND FUTURE PERSPECTIVES

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.[96–99] 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 all 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.[96–99]

A general concern in case of fluorescence studies in motionally restricted systems is that red edge effects could complicate results if such fluorophores are excited at the red edge of the absorption spectra rather than at the absorption maxima. Thus, studies such as energy transfer could lead to significant errors unless carried out with caution. 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.[100] In general, a decrease in the apparent depth of the fatty-acyl-attached probes was found both at longer excitation wavelengths and at longer emission wavelengths.

Another potential complication may 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.[101,102] 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 the red edge 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[103] as well as integral membrane[104] proteins. A major limitation in working with multitryptophan proteins is that the analysis of fluorescence is often complicated due to the complexity of fluorescence processes in such systems.[105,106] *Site-specific* incorporation of ex-

trinsic probes, very recently accomplished by using unnatural amino acid mutagenesis,[107,108] 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.

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