

NOVEL INSIGHTS INTO PROTEIN STRUCTURE AND DYNAMICS UTILIZING THE RED EDGE EXCITATION SHIFT APPROACH

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ABSTRACT

A shift in the wavelength of maximum fluorescence 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). This effect is mostly observed with polar fluorophores in motionally restricted media such as 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 depends on 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 extremely useful since hydration plays a crucial modulatory role in the formation and maintenance of organized molecular assemblies such as folded proteins in aqueous solutions and biological membranes. The application of REES as a powerful tool to monitor the organization and dynamics of a variety of soluble, cytoskeletal, and membrane-bound proteins is discussed.

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9.1. INTRODUCTION

Proteins are highly ordered, dynamic, complex biological macromolecules involved in many cellular functions in their native state. In order to understand the structure-function relationship in biological macromolecules such as proteins, it is necessary to appreciate the dynamics of the constituent molecules as well as that of the system as a whole. Water plays a crucial role in determining the structure and the dynamics, and in turn the functionality of proteins.¹⁻¹¹ Protein-water interactions are therefore vital to biological functions.¹² It is estimated that a threshold level of hydration (less than 0.4 grams of water per gram of protein) is required to fully activate the dynamics and function of globular proteins.¹³⁻¹⁵ The most direct evidence for the importance of water in protein structure and function is that in the absence of water, proteins cannot diffuse and become non-functional. Lack of motion and function have been observed when proteins are transferred to organic solvents¹⁶ and dehydration studies show that at least a monolayer of water molecules is required for the protein to be fully functional.¹⁷ These studies not only prove that the presence of water is essential for the organization and function of proteins, but also that other solvents cannot serve as substitutes. In addition, it has become increasingly evident that water molecules mediate lipid-protein interactions¹⁸⁻²⁰ and hence the function of membrane proteins.²¹⁻²³

Although detailed and precise structural information of proteins, particularly soluble proteins, can be obtained from x-ray crystallographic diffraction data, such information is necessarily static. Interestingly, global and local dynamics exhibited by proteins and specific regions in them play important roles in their function.^{24, 25} Further, a detailed crystallographic database is still not available in the case of membrane proteins due to the inherent difficulty in crystallizing them.^{26, 27} This is apparent when one considers that the number of membrane proteins with known x-ray crystal structures is still very small and represents only ~0.2% of all solved protein structures.²⁷⁻³¹ Spectroscopic techniques which provide both structural and dynamic information therefore become very useful for analyses of proteins. Fluorescence spectroscopy represents one such approach and is widely used in the analysis of protein structure, dynamics and function. The advantages of using fluorescence techniques are intrinsic sensitivity, suitable time scale, non-invasive nature, and minimum perturbation.^{32, 33} This review is focussed on the application of a relatively novel approach, the red edge excitation shift (REES), as a powerful tool to monitor the organization and dynamics of proteins and peptides.

REES represents a powerful approach which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system.³⁴⁻³⁹ 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 REES. This effect is mostly observed with polar fluorophores in motionally restricted media such as 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 depends on 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 extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events.⁴⁰ The application of REES to elucidate the organization and dynamics of membranes^{36, 38} and membrane-bound peptides and proteins has been reviewed recently.³⁹

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.^{41, 42} It is obvious from this rule that fluorescence emission should be independent of excitation wavelength. 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. 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).³⁵⁻³⁹ 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. 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. Since the dipole moment of a molecule changes upon excitation, the solvent dipoles have to reorient around this new excited state dipole 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 due to 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 therefore a much slower process and is dependent on the restriction to their mobility offered by the surrounding matrix. More precisely, for a polar fluorophore

in a bulk non-viscous solvent, this reorientation occurs at a time scale 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, which is typically of the order of 10^{-9} sec. 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 to 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 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 shell 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 REES can therefore be summarized as follows: (i) The fluorophore should normally be polar so as to be able to suitably orient the neighboring solvent molecules in the ground state (molecules such as bianthryl which are nonpolar in the ground state and yet could be polar in the excited state due to intramolecular charge transfer reaction are exceptions to this⁴³⁻⁴⁵); (ii) The solvent molecules surrounding the fluorophore should be polar; (iii) The solvent reorientation time around the excited state dipole of the fluorophore should be comparable to or longer than the fluorescence lifetime; 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). It has previously been shown for tryptophan, the most commonly found intrinsic fluorophore in proteins, that a dipole moment change of ~ 6 D upon excitation^{46, 47} is enough to give rise to significant red edge effects. A recent comprehensive review on REES is provided by Demchenko.³⁷

9.3. INTRINSIC FLUORESCENCE OF PROTEINS AND PEPTIDES: TRYPTOPHAN AS THE FLUOROPHORE OF CHOICE

The aromatic amino acids tryptophan, tyrosine and phenylalanine are capable of contributing to the intrinsic fluorescence of proteins. When all three residues are present in a protein (termed as class B protein),⁴⁸ pure emission from tryptophan can be obtained only by photoselective excitation at wavelengths above 295 nm.³² Although tyrosine and phenylalanine are natural fluorophores in proteins, tryptophan is the most extensively used amino acid for fluorescence analysis of proteins. In a protein containing all three naturally fluorescent amino acids, observation of tyrosine and phenylalanine fluorescence is often complicated because of interference by tryptophan due to resonance energy

transfer.^{32, 49} The application of tyrosine and phenylalanine fluorescence is therefore mostly limited to tryptophan-free proteins (however, a recent study reports an exception to this⁵⁰). More importantly, tyrosine fluorescence is insensitive to environmental factors such as polarity and does not exhibit appreciable solvatochromism in sharp contrast to tryptophan fluorescence.⁵¹ This is a clear disadvantage for a fluorescent reporter group in biological applications. Fluorescence of phenylalanine is weak and seldom used in protein studies.⁴⁹ Hence, the term 'natural protein fluorescence' is almost always associated with tryptophan fluorescence.⁵²

Tryptophan residues serve as intrinsic, site-specific fluorescent probes for protein structure and dynamics³² and are generally present at about 1 mol% in proteins.⁴⁹ The low tryptophan content of proteins is a favorable feature of protein structure since a protein may typically possess few tryptophan residues which facilitate interpretation of fluorescence data and avoid complications due to inter-tryptophan interactions. The well documented sensitivity of tryptophan fluorescence to environmental factors such as polarity and mobility makes tryptophan fluorescence a valuable tool in studies of protein structure and dynamics by providing specific and sensitive information of protein structure and its interactions.^{32, 49, 53, 54} The presence of tryptophan residues as intrinsic fluorophores in most peptides and proteins makes them an obvious choice for fluorescence spectroscopic analysis as apparent from the fact that ~300 papers utilizing tryptophan fluorescence in proteins are published per year.⁵⁵

The interesting spectral properties of tryptophan are attributed to a number of factors. The tryptophan residue has a large indole side chain that consists of two fused aromatic rings (see Figure 1). Tryptophan has two overlapping $S_0 \rightarrow S_1$ electronic transitions denoted as 1L_a and 1L_b which are almost perpendicular to each other.³² Both $S_0 \rightarrow ^1L_a$ and $S_0 \rightarrow ^1L_b$ transitions occur in the 260-300 nm range. In nonpolar solvents, 1L_a has higher energy than 1L_b . In polar solvents, however, the energy level of 1L_a is lowered making it the lowest energy state. This inversion is believed to occur because 1L_a transition has a higher dipole moment (as it is directed through the -NH group of the

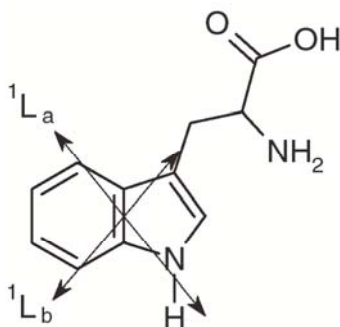


Figure 9.1. Chemical structure of tryptophan showing the transition moment directions for the 1L_a and 1L_b transitions. The 1L_a is the fluorescing state in most proteins and has higher dipole moment (as it is directed through the ring -NH group), and can have dipole-dipole interactions with polar solvent molecules. In nonpolar solvents, 1L_a has higher energy than 1L_b . However, the energy level of 1L_a is lower in polar solvents due to favorable dipole-dipole interactions making it the lowest energy state. Adapted and modified from ref. 32.

indole ring), and can have dipole-dipole interactions with polar solvent molecules. Irrespective of whether 1L_a or 1L_b is the lowest S_1 state, equilibration between these two states is believed to be very fast (of the order of 10^{-12} sec), so that emission only from the lower S_1 state is observed.⁵⁶ It is generally believed that 1L_a is the fluorescing state in all proteins with the possible exception of Trp-48 of azurin.⁵⁵ In molecular terms, tryptophan is a unique amino acid since it is capable of both hydrophobic and polar interactions. This is due to the fact that while tryptophan has the polar $-NH$ group which is capable of forming hydrogen bonds, it also has the largest nonpolar accessible surface area among the naturally occurring amino acids.⁵⁷ Due to its aromaticity, the tryptophan residue is capable of $\pi-\pi$ interactions and weakly polar interactions.^{58, 59} This amphipathic character of tryptophan gives rise to its unique hydrogen bonding property and ability to function through long range electrostatic interaction.⁶⁰ The amphipathic character of tryptophan also explains its interfacial localization in membranes^{61, 62} which is characterized by unique motional and dielectric characteristics different from the bulk aqueous phase and the more isotropic hydrocarbon-like deeper regions of the membrane (see later). More importantly, apart from the structural and spectral properties, the role of tryptophan residues in maintaining the structure and function of both soluble⁶³⁻⁶⁵ and membrane^{60, 66-72} proteins has attracted considerable attention. The importance of tryptophan residues is exemplified by the fact that any perturbation to tryptophan residues (substitution, deletion, chemical modification, or photodamage) often results in reduction or loss of protein functionality.

9.4. APPLICATION OF REES IN THE ORGANIZATION AND DYNAMICS OF PROTEINS

9.4.1. Soluble Proteins

The local dynamics of the protein matrix around a given amino acid residue can be examined from the rate at which the matrix responds to (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. The first protein for which REES was documented using its intrinsic fluorescence was human serum albumin.⁷³ Pioneering work in the application of REES to elucidate protein organization and dynamics was carried out by Demchenko and co-workers (recently reviewed in ref. 37). In one of their early studies, it was shown that the fluorescence emission spectra of 2-(*p*-toluidinylnaphthalene)-6-sulfonate (TNS) bound to 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.⁷⁴ 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.

Based on an in-depth study of a variety of single tryptophan proteins, it was observed that the presence of red edge effects in such proteins is influenced by the position of emission maximum of tryptophan fluorescence when excited at the absorption maximum.⁷⁵ It was found that proteins (such as azurin, parvalbumin, and ribonucleases

T₁ and C₂) with maximum of emission at very short wavelengths (*i.e.*, between 307 and 323 nm) do not show red edge effects. This is attributed to the fact that the tryptophan environments in this class of proteins are nonpolar and the dipole-orientational broadening of the spectra is insufficient to create a large enough distribution of differentially solvated substrates that could be photoselected in order to give rise to REES. However, a recent study provides an exception to this generalization in which a single tryptophan mutant of triosephosphate isomerase from the parasite *Plasmodium falciparum*, with an emission maximum of 321 nm, has been shown to exhibit REES.⁷⁶ Interestingly, REES was also not observed for proteins (such as myelin basic protein, β -casein, and monomeric melittin in water) with long wavelength emission maximum (beyond 341 nm). The tryptophan residue in these cases is exposed to aqueous environment undergoing much faster solvent relaxation as compared to the fluorescence lifetimes of the concerned tryptophans. It is for this reason that tryptophans in denatured proteins do not exhibit REES, *e.g.*, tubulin⁷⁷ (see later). However, considerable REES effects were observed for many proteins (such as tetrameric form of melittin, human serum albumin, albumin complexed with sodium dodecyl sulfate) with intermediate maxima of emission (between 325 and 341 nm). For these proteins, there is enough range of dipole-orientational broadening and at the same time, the rate of solvent reorientation is slow enough to give rise to REES. It is interesting to note that monomeric melittin in aqueous solution does not exhibit REES whereas the tetrameric form of melittin exhibits a REES of 6 nm indicating that the tryptophan residues are in a motionally restricted environment in the aggregated form of melittin.⁷⁸ However, monomeric melittin has been shown to exhibit significant REES upon membrane binding (see section 4.2.3).

While human serum albumin shows REES in the absence of detergents,⁷⁵ the emission maximum of bovine serum albumin is independent of the excitation wavelength. However, upon binding to detergents, there is an increase in solvent restriction around the tryptophan residues in both human and bovine serum albumins, as evident by the enhanced red edge effects.^{75, 79} Interestingly, the presence of slow solvent relaxation at ambient temperature has been demonstrated utilizing REES of the single tryptophan protein Bj2S, a seed albumin from *Brassica juncea*.⁸⁰ The REES approach has been used in a number of cases to monitor the tryptophan environment and dynamics in proteins such as human α_1 -acid glycoprotein,⁸¹ bothropstoxin-I from the venom of *Bothrops jararacussu*,⁸² ascorbate oxidase,⁸³ smooth muscle myosin light chain kinase,⁸⁴ skeletal myosin rod,⁸⁵ the human leukocyte antigen complex,⁸⁶ and the pore-forming α -toxin from *Staphylococcus aureus*.⁸⁷ In addition, it has been shown that the environment of tryptophans in cytoskeletal proteins such as tubulin⁷⁷ and spectrin^{88, 89} are motionally restricted and display REES. Importantly, observation of REES in multityryptophan proteins considerably rules out the possibility of homotransfer among tryptophans.⁹⁰

In addition to the application of REES to monitor protein conformation in solution, it has also been applied to more complex systems such as intact eye lens and cornea. About 35% of the lens (by weight) is made up of three closely related proteins called crystallins. 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.⁹¹ The use of REES has also been extended to relate the properties of the lens with those of its constituent proteins and

their homo- and heteroaggregates⁹² and of proteoglycans and crystallins from intact cornea.^{93, 94}

9.4.1.1. REES as a Tool to Explore Residual Local Structure in Denatured Proteins

As mentioned earlier, tryptophans in denatured proteins generally do not exhibit REES due to fast solvent relaxation in the denatured state. For example, tryptophans in the cytoskeletal protein tubulin show REES of 7 nm. However, no REES is observed when the protein is denatured in 8 M urea.⁷⁷ In a recent study on the cytoskeletal protein erythroid spectrin, the tryptophans show REES indicating the localization in environments which are motionally restricted due to slow solvent relaxation.^{88, 89} Interestingly, spectrin display REES even when denatured in 8 M urea.⁸⁸ This surprising result is in contrast to earlier studies where it was shown that the emission maximum of tryptophans in denatured proteins do not exhibit excitation wavelength dependence. This is because the tryptophans are exposed to water when denatured and therefore do not offer any restriction to the solvent (water) dipoles around them in the excited state.⁷⁵ The observation of REES in denatured spectrin indicates that the tryptophans are shielded from bulk solvent even when denatured and indicates local residual structure in the denatured protein. The tryptophan microenvironment in spectrin is, therefore, characterized by unique structural and dynamic features that are maintained to a significant extent even when denatured with urea. This is further supported by analysis of fluorescence quenching data using acrylamide as quencher.⁸⁸ Such residual structure in an unfolded protein is thought to reside predominantly in hydrophobic clusters, where residues like tryptophan stabilize these networks through cooperative long range nonnative interactions.⁹⁵ Although residual structure in denatured proteins has been studied before,^{96, 97} this example constitutes the first demonstration of slow solvent relaxation in a completely denatured protein. In addition, the influence of ionic strength induced conformational change of spectrin on the solvent-restricted environment of the tryptophan residues has been recently addressed.⁸⁹

4.2. Membrane Peptides and Proteins

9.4.2.1. Membrane Interface: An Appropriate System for the REES Approach

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. Organized molecular assemblies such as membranes can be considered as large cooperative units with characteristics very different from the individual structural units that constitute them. A direct consequence of such highly organized systems is the restriction imposed on the mobility of their constituent structural units. It is well known that interiors of biological membranes are viscous, with the effective viscosity comparable to that of light oil.⁹⁸⁻¹⁰⁰ In addition, membranes exhibit considerable degree of anisotropy along the axis perpendicular to the bilayer. While the center of the bilayer is nearly isotropic, the upper portion, only a few

angstroms away toward the membrane surface, is highly ordered.^{38, 101-105} Properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds and extent of solvent penetration vary in a depth-dependent manner in the membrane. The interfacial region in membranes is the most important region so far as the dynamics and function of the membrane is concerned. The membrane interface is characterized by unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the more isotropic hydrocarbon-like interior of the membrane.^{101-104, 106} It is a chemically heterogeneous region composed of lipid headgroup, water and portions of the acyl chain.¹⁰⁷ Overall, the interfacial region of the membrane accounts for 50% of the thermal thickness of the bilayer.¹⁰⁶ This specific region of the membrane is known to exhibit slow solvent (water) relaxation and participate in intermolecular charge interactions¹⁰⁸ and hydrogen bonding through the polar headgroup.¹⁰⁹⁻¹¹¹ These structural features, which slow down the rate of solvent reorientation, have previously been recognized as typical features of environments giving rise to significant red edge effects.^{38, 112} It is therefore the membrane interface which is most likely to display REES.^{105, 113-115} The application of REES to study the organization and dynamics of membranes has been reviewed recently.^{36, 38}

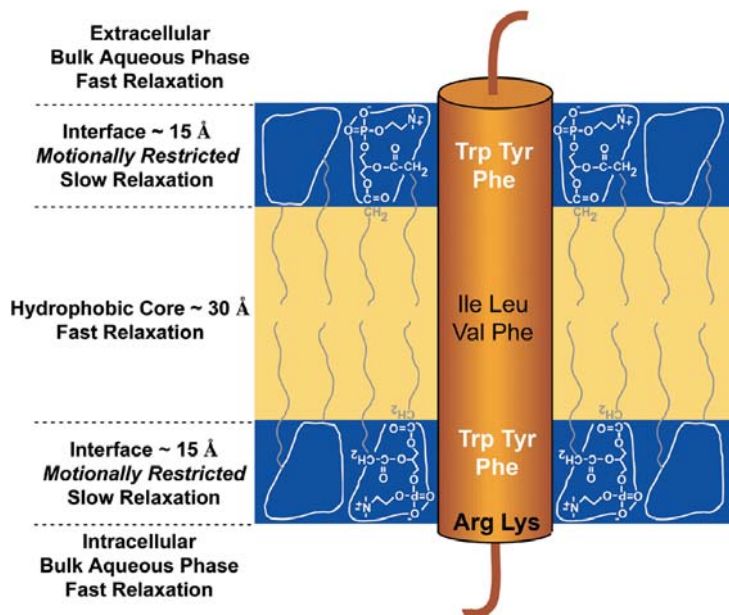


Figure 9.2. A schematic representation of the membrane showing the various regions of the bilayer displaying motional anisotropy. The membrane lipids shown have two hydrophobic tails with a phosphatidylcholine (PC) headgroup. The preferred locations of various amino acids present in a membrane-spanning transmembrane domain are also shown. It should be noted that the tryptophan residues are localized in the membrane interface region, a region characterized by unique organization, dynamics, hydration and functionality. Adapted from ref. 39.

9.4.2.2. *Tryptophan and the Membrane Interface*

The biological membrane provides a unique environment to membrane-spanning proteins and peptides influencing their structure and function. Membrane-spanning proteins have distinct stretches of hydrophobic amino acids that form the membrane-spanning domain and are reported to have a significantly higher tryptophan content than soluble proteins.¹¹⁶ In addition, it has been observed 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 bonding¹¹⁷ with the lipid carbonyl groups or interfacial water molecules (see Figure 2). For instance, crystal structures of membrane proteins such as the potassium channel,¹¹⁸ bacteriorhodopsin,¹¹⁹ maltoporin,¹²⁰ 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.¹²¹⁻¹²³ Furthermore, for transmembrane peptides and proteins, tryptophan has been found to be an efficient anchor at the membrane interface^{116, 124} and defines the hydrophobic length of transmembrane helices.¹²⁵ Importantly, the role of tryptophan residues in maintaining the structure and function of membrane proteins is exemplified by the fact that substitution or deletion of tryptophans often results in reduction or loss of protein functionality.^{60, 66, 69}

The exact location and orientation of tryptophan residues at the membrane interface is, however, not clear. Some experiments suggest that tryptophan residues have a preference for the lipid headgroup side of the interface but others suggest that the preference is for the fatty acyl chain side.¹²⁶⁻¹²⁹ Nevertheless, 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.¹²⁶ The tryptophan rich aromatic belt at the membrane interface in transmembrane helices is thought to stabilize the helix with respect to the membrane environment.¹²² The tryptophan residue has a large indole side chain that consists of two fused rings. In fact, the tryptophan side chain has the largest volume of all the amino acid side chains,¹³⁰ with a volume of 228 Å³ which is comparable to the volume of a phosphatidylcholine headgroup,¹³¹ *i.e.* 319 Å³. Wimley and White have shown from partitioning of model peptides to membrane interfaces that the experimentally determined interfacial hydrophobicity of tryptophan is the highest among the naturally occurring amino acid residues thus accounting for its specific interfacial localization in membrane-bound peptides and proteins.⁶¹ 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 interactions.^{60, 132} This 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 favorable electrostatic interactions and hydrogen bonding.

9.4.2.3. *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 represent convenient molecules to explore the specific role of membrane and peptide structural properties on the orientation, incorporation, stability and function of such peptides in the membrane milieu.¹³³⁻¹³⁹

Tryptophan octyl ester (TOE) has been recognized as an important model for membrane-bound tryptophan residues.^{62, 140-142} 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.⁶²

Melittin, the major toxic component in the venom of the European honey bee, *Apis mellifera*, was one of the earliest membrane peptides studied utilizing REES. It is an amphipathic cationic hemolytic peptide with a single, functionally important tryptophan residue.¹⁴³ The amphipathic nature is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins.^{143, 144} This has made melittin very popular as a model to study lipid-protein interactions in membranes.^{143, 145} Results from studies utilizing REES of the sole tryptophan residue showed that when bound to zwitterionic membranes, the microenvironment of the functionally active tryptophan of melittin is motionally restricted, consistent with the interfacial location of the tryptophan residue.¹⁴⁶ Interestingly, further results employing REES 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 these two cases.¹⁴⁷ REES is therefore sensitive to changes in dynamics of hydration caused by varying electrostatic interactions. This has been recently confirmed using melittin bound to micelles of various charge types.¹⁴⁸ In addition, the REES approach has recently been successfully applied to monitor the effect of membrane cholesterol on the organization and dynamics of melittin.¹⁴⁹

Reverse micelles serve as appropriate membrane-mimetic host assembly for the characterization of membrane-active peptides and proteins utilizing REES.¹⁵⁰ This is due to the fact that reverse micelles offer the unique advantage of monitoring dynamics of embedded molecules with varying degrees of hydration which is difficult to achieve with complex systems such as membranes. It has thus been shown that the magnitude of REES for melittin bound to reverse micelles is sensitive to the change in water content of the system.¹⁵¹ This constitutes the first report directly demonstrating that REES is sensitive to the changing dynamic hydration profile of an amphiphilic peptide. In another study, the magnitude of REES of membrane-bound melittin has been shown to be dependent on the extent of lipid chain unsaturation in the membrane. It is interesting to note that the magnitude of REES obtained with melittin in membranes containing more than two double bonds is considerably higher (up to 19 nm)¹⁵² than what is usually reported for membrane-bound tryptophan residues,^{62, 146, 147, 153-156} although higher REES has been reported in a few cases (see below). In addition, the environment of tryptophan residues of several tryptophan-rich antimicrobial peptides in the membrane-bound form has been explored utilizing REES.¹⁵⁷

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 probe the localization and dynamics of

the functionally important tryptophan residues in the gramicidin channel.^{153, 156} Gramicidin belongs to a family of prototypical cation-selective channel formers, which are naturally fluorescent due to the presence of four tryptophan residues.¹⁵⁸ These interfacially localized tryptophans are known to play a crucial role in the organization and function of the channel.^{60, 66} The results from these studies point out the motional restriction experienced by the tryptophans at the peptide-lipid interface of the gramicidin channel.^{153, 156} This is consistent with other studies^{60, 66} in which such a restriction is 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.^{60, 66, 159-163} More importantly, REES and related fluorescence approaches have been used to distinguish between the channel and non-channel conformations of gramicidin in membranes.¹⁵⁶ REES of gramicidin is therefore sensitive to the conformation adopted by the peptide in the membrane. This provides a convenient spectroscopic handle to monitor the functional status of this important ion channel peptide. In addition, REES has been used to monitor the effects of structural transition of the host assembly and hydration on the organization and dynamics of gramicidin.^{164, 165} Further, REES of gramicidin has been found to be dependent on the lipid composition¹⁶⁶ and phase state of the membrane.¹⁶⁷

In the absence of crystal structures, fluorescence approaches to study the topography and membrane interactions of specific peptide fragments have proved to be very useful. REES has been used to probe the membrane interaction of synthetic peptides corresponding to the functionally important regions of membrane binding proteins.^{168, 169} The 579-601 fragment of the ectodomain of the HIV-1 gp-41 protein which is essential for its activity, was shown to be incorporated in model membranes.¹⁶⁸ Upon red edge excitation, there was a substantial red shift in fluorescence emission 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 tryptophan residues which emit between 325 and 341 nm.⁷⁵ 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 ω -loop region of the human prothrombin γ -carboxyglutamic acid domain.¹⁶⁹ In both these cases, the peptides are found to partition to the interfacial region of the membrane. The observed REES would therefore be a direct consequence of the motionally restricted membrane interface.³⁸ In yet another study, the organization and topography of the membrane-binding C2 domain of factor VIII have been monitored using REES in membrane-mimetic environments.¹⁷⁰

The interaction of the peptide corresponding to the γ M4 transmembrane domain of the nicotinic acetylcholine receptor with membranes has been investigated.¹⁷¹ Studies using REES 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 observation that γ M4 forms a transmembrane helix.¹⁷² The application of REES has been extended in a novel way to determine the topology and

membrane localization of tryptophan residues in the colicin E1 channel peptide by measuring REES of single tryptophan containing channel peptides in the soluble aqueous state, and when bound to membranes.¹⁵⁴ 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.

9.4.2.4. Membrane Proteins

An early application of REES 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.¹⁵⁵ 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 the membrane. In another study, the tryptophans of the pore-forming *Staphylococcus aureus* α -toxin were shown to exhibit REES.⁸⁷ However, no significant difference was observed between REES displayed by the soluble and membrane-bound forms of the toxin indicating no drastic change in the tryptophan environment upon membrane binding. The tryptophans in *E. coli* porin Omp-F, a pore-forming channel protein, also exhibit REES.¹⁷³

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 membrane-active protein is the mitochondrial creatine kinase which shows a large increase in REES upon membrane binding.¹⁷⁴ 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,¹⁷⁵ the observed motional restriction has been attributed to conformational changes of the protein induced by lipid binding.

9.4.3. Extrinsic Fluorophores

The analysis of fluorescence from multityryptophan proteins is often complicated due to the complexity of fluorescence processes in such systems, and the heterogeneity in fluorescence parameters (such as quantum yield and lifetime) due to environmental sensitivity of individual tryptophans.³² This problem can be avoided by the use of extrinsic fluorophores which often display improved spectral properties. Such extrinsic fluorescent probes are widely used to study the dynamics of proteins and membranes.^{74, 105, 113, 114, 176} The advantage of this approach is that one has a choice of the fluorescent label to be used and, therefore, specific probes with appropriate characteristics can be designed for specific applications. Many of these probes display high sensitivity to the polarity of the local environment. In addition, many extrinsic fluorescent probes are weakly fluorescent or nonfluorescent in water but fluoresce strongly when bound to proteins and membranes making the contribution from the unbound probe negligible.

Fluorescent probes such as 6-(*p*-toluidinyl)-2-naphthalenesulfonic acid (TNS) and 8-anilino-naphthalene-1-sulfonic acid (ANS) are representative examples of this type of probes. These probes are considered as indicators of binding site polarity, and the shifts

in fluorescence spectra on binding are commonly correlated with solvent polarity scales. For example, by comparing data obtained for TNS in solvents and bound to macromolecules, it has been found that there is a slower relaxation around TNS bound to the heme site of the apomyoglobin.¹⁷⁶ As mentioned earlier, in one of the early studies of REES, it was shown that TNS bound to proteins such as β -lactoglobulin, β -casein, and bovine and human serum albumins exhibits REES of the order of 10 nm.⁷⁴ In addition, the complexes of TNS with tetrameric melittin¹⁷⁷ and α_1 -acid glycoprotein¹⁷⁸ demonstrated that the TNS-binding site is rigid on the nanosecond time scale in these proteins giving rise to REES.

The hydrophobic fluorescent probe 6-propionyl-2-dimethylaminonaphthalene (PRODAN) shows polarity-sensitive fluorescence.¹⁷⁹ The dipole moment of PRODAN changes by ~ 5 -8 D upon excitation.^{180, 181} A change in dipole moment of this magnitude, along with its hydrogen bonding capability,¹⁸¹ makes PRODAN a suitable probe for monitoring REES effects to characterize hydrophobic binding sites in proteins. For example, PRODAN binds erythroid spectrin with a high affinity.¹⁸² The organization and dynamics of the PRODAN binding site in erythroid spectrin have been monitored recently.⁸⁸ The observation of REES for spectrin-bound PRODAN suggests that PRODAN is in an environment where its mobility is considerably reduced. Since PRODAN binds to the hydrophobic site in spectrin, this result shows that this region offers considerable restriction to the reorientational motion of the solvent dipoles around the excited state fluorophore.⁸⁸ In addition, pronounced REES was reported for complexes of PRODAN with model lipoproteins.¹⁸³ Interestingly, a derivative of PRODAN, 2'-(*N,N*-dimethyl)amino-6-naphthoyl-4-*trans*-cyclohexanoic acid (DANCA), has been used to determine the polarity of the heme-binding pocket in apomyoglobin utilizing the excitation wavelength dependence of the emission spectrum.¹⁸⁴

In an attempt to understand the dynamics of ligand-binding pockets in proteins, REES studies have also been carried out utilizing naturally fluorescent ligands. For instance, bilirubin is a natural ligand for the human and bovine serum albumins. It has been shown to exhibit REES when complexed with albumins in the presence of micellar concentrations of Triton X-100 which confer rigidity to the binding pocket of bilirubin in albumins.⁷⁹ In another study, REES of the antimetabolic drug colchicine and its derivatives was detected when bound to tubulin using colchicine fluorescence in order to understand structure-function relationship in the tubulin-colchicine complex.⁷⁷ In addition, REES of flavin adenine dinucleotide (FAD) bound to lipoamide dehydrogenase from *Azotobacter vinelandii* has also been reported.¹⁸⁵ Taken together, these results show that REES can be conveniently used to monitor the organization and dynamics of ligand-binding pockets of proteins.

A widely used extrinsic probe is the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group which reacts with amino or thiol groups to form stable, highly fluorescent compounds.¹⁸⁶ In this way, various NBD-labeled lipids and proteins have been generated and these have proved to be useful for monitoring structure and dynamics of proteins, peptides, and membranes. NBD-labeled lipids are extensively used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes. The NBD moiety possesses some of the most desirable properties for serving as an excellent probe for spectroscopic applications. It is very weakly fluorescent in water and is relatively photostable. Upon transfer to a hydrophobic medium, it fluoresces brightly in the visible

range and exhibits a high degree of environmental sensitivity.^{113, 187-190} More importantly, it has earlier been shown using solvatochromic and quantum chemical approaches that the dipole moment of the NBD group changes by ~ 4 D upon excitation,¹⁸⁹ an important criterion for a fluorophore to exhibit REES effects.^{35, 38} The NBD group therefore has been used extensively to explore the organization and dynamics in membranes and membrane-mimetic media utilizing the REES approach.^{113, 114, 191-197}

Apolipoprotein C-II (apoC-II) is an exchangeable 79-residue apolipoprotein found in blood plasma and is essential for the activation of lipoprotein lipase and therefore in the hydrolysis of triacylglycerols and the prevention of hypertriglyceridaemia.¹⁹⁸ By covalently labeling the peptide fragment of apolipoprotein C-II (apoC-II₁₉₋₃₉) with NBD, it was found that NBD-apoC-II₁₉₋₃₉ exhibits an increased REES of 25 nm when bound to egg yolk phosphatidylcholine membranes when compared to the corresponding REES in aqueous solution (9 nm).¹⁹⁹ This observation, along with results from time-resolved fluorescence measurements, points out that the NBD-labeled peptide is located near the polar-interfacial region of the bilayer where it experiences a heterogeneous environment. In addition, the newly developed approach of site-directed fluorescence labeling (SDFL)^{200, 201} at a single site on the target protein, could be extremely useful to monitor the environmental dynamics of proteins in a site-specific manner (see later), particularly for multitryptophan containing proteins.

9.5. CONCLUSION AND FUTURE PERSPECTIVES

The lack of a suitable fluorophore often makes it difficult to monitor the dynamics in a region of interest in proteins. Advances in molecular biological techniques, however, have made it possible to incorporate (or substitute) endogeneous labels such as tryptophan in regions of choice in soluble²⁰² as well as integral membrane²⁰³ proteins. A major limitation in working with multitryptophan proteins is that the analysis of fluorescence data is often complicated due to the complexity of fluorescence processes in such systems and lack of specific information.^{32, 204} Site-specific incorporation of extrinsic probes, accomplished by using unnatural amino acid mutagenesis,²⁰⁵⁻²⁰⁷ could help avoid this complication. The incorporation of tryptophan analogues has been shown to be useful in this regard. Successful biosynthetic incorporation of tryptophan analogues has been accomplished with various derivatives of azatryptophan, hydroxytryptophan, and fluorotryptophan.²⁰⁸ The main advantage of these analogues is that the spectral properties are distinct from that of tryptophan. For example, the absorption and fluorescence emission spectra of 7-azatryptophan are significantly red shifted (by 10 nm and 50 nm respectively) from those of tryptophan.²⁰⁹ Consequently, it is possible to selectively excite these tryptophan analogues in proteins, in the presence of a 'sea' of tryptophans of other proteins in their native environment. More importantly, it has been shown that the incorporation of these analogues causes minimal perturbation to protein structure, stability, and function.²¹⁰⁻²¹² Interestingly, 7-azatryptophan has been shown to display REES and is sensitive to slow solvent relaxation at low temperatures,²¹³ which makes it an ideal candidate to explore site-specific environmental rigidity in proteins.

As mentioned earlier, water plays a crucial role in the formation and maintenance of organized molecular assemblies such as proteins and membranes in a cellular environment. Knowledge of dynamics of hydration at the molecular level is therefore of

considerable importance in understanding the cellular structure and function.^{3, 12, 15, 21-23, 40, 214-218} 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. 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. Since the dynamics of hydration is directly associated with the functionality of proteins, REES in combination with the molecular biological approaches for incorporating novel fluorophores at specific sites, could prove to be a novel and extremely powerful tool to explore the organization and dynamics of both soluble and membrane proteins and other organized molecular assemblies.

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