Application of fluorescence spectroscopy to membrane protein structure and dynamics

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In spite of the functional importance of membrane proteins, information on their structure and organization is lacking due to the paucity of crystal structures. In the absence of a detailed crystallographic database, approaches based on fluorescence spectroscopy have proved useful in elucidating the organization, topology and orientation of membrane proteins. This review is focussed on the application of various approaches based on fluorescence spectroscopy to explore the organization and dynamics of membrane proteins and peptides. Some of the important approaches include analysis of depth of penetration of membrane proteins and peptides utilizing fluorescence quenching, site-directed fluorescence labeling and the wavelength-selective fluorescence approach.

Membrane proteins: the unconquered battle for the structural biologist

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. Due to both lipid–protein and protein–protein interactions, the biological membrane constitutes the site of many important cellular functions such as energy metabolism, muscle contraction, nutrient absorption, signal transduction, ion transport, cell–cell contact and recognition[1]. However, our understanding of these processes at the molecular level is limited by the lack of high resolution three-dimensional structures of membrane-bound proteins and peptides. This is in spite of the fact that about 30–40% of all proteins are integral membrane proteins[2]. For example, ~30% of the proteins coded by the human genome are membrane proteins. Interestingly, it is estimated that ~60% of drug targets in the pharmaceutical industry are membrane proteins[3]. Knowledge of the structure and organization of membrane proteins therefore represents a major step toward understanding the function of membrane proteins.

In spite of some recent successes[4–6], crystallization of membrane proteins and peptides for diffraction studies continues to be extremely difficult and challenging. Although the first complete X-ray crystallographic analysis of an integral membrane protein was successfully carried out a number of years back[7], the number of membrane proteins whose X-ray crystal structures are known is still very small and represents only ~0.2% of all solved protein structures[8]. Although detailed and precise structural information of proteins (particularly soluble proteins) can be obtained from crystallographic diffraction data, such information is necessarily static. However, global and local dynamics exhibited by proteins and specific regions in them play important roles in their function. Further, a detailed crystallographic database is still not available in case of membrane proteins and peptides due to the inherent difficulty in crystallizing them. The great disparity between our understanding of soluble proteins and membrane proteins is a consequence of many practical problems of working with membrane proteins. Even high resolution NMR methods have limited applications for membrane-bound proteins and peptides because of slow reorientation times in membranes[9].

For this reason, most structural analyses of such molecules have utilized other biophysical techniques with an emphasis on spectroscopic approaches. Spectroscopic techniques, which provide both structural and dynamic information, therefore become very useful for analyses of membrane proteins. Fluorescence spectroscopy represents one such approach and is widely used in analysis of membrane protein structure and function. The advantages of using fluorescence techniques are intrinsic sensitivity, suitable time scale, non-invasive nature, and minimum perturbation[10–15]. In addition, the ability to incorporate fluorophores in a site-specific manner makes fluorescence approaches very powerful[16]. This review is focussed on the application of various approaches based on fluorescence spectroscopy to explore the organization and dynamics of membrane proteins and peptides.

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 the class B protein), pure emission from tryptophan can be obtained only by photoselective excitation at wavelengths above 295 nm (ref. 17).

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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 fluorescent amino acids, observation of tyrosine and phenylalanine fluorescence is often complicated due to the interference by tryptophan by resonance energy transfer[17,18]. The application of tyrosine and phenylalanine fluorescence is therefore mostly limited to tryptophan-free proteins (however, a recent study reports an exception to this[19]). More importantly, tyrosine fluorescence is insensitive to environmental factors such as polarity and does not exhibit appreciable solvatochromism in sharp contrast to tryptophan fluorescence[20]. This is a clear disadvantage for a fluorescent reporter group in biological applications. Fluorescence of phenylalanine is weak and seldom used in protein studies[18]. Hence, the term 'natural protein fluorescence' is almost always associated with tryptophan fluorescence[21].

Tryptophan residues serve as intrinsic, site-specific fluorescent probes for protein structure and dynamics[17] and are generally present at about 1 mol% in proteins[18]. The low tryptophan content of proteins is a favourable 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 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[17,18]. The presence of tryptophan residues as intrinsic fluorophores in most peptides and proteins therefore makes them an obvious choice for fluorescence spectroscopic analysis.

Role of tryptophan residues in membrane proteins and peptides: tryptophan and the membrane interface

The role of tryptophan residues in the structure and function of membrane proteins has recently attracted a lot of attention[22–24]. The biological membrane provides a unique environment to membrane-spanning proteins and peptides thus influencing their structure and function. Membrane-spanning proteins have distinct stretches of hydrophobic amino acids that form the membrane-spanning domain and have been reported to have a significantly higher tryptophan content than soluble proteins[25]. In addition, it is becoming increasingly evident that tryptophan residues in integral membrane proteins and peptides are not uniformly distributed and that they tend to be localized toward the membrane interface, possibly because they are involved in hydrogen bonding[26] with the lipid carbonyl groups or interfacial water molecules (see Figure 1). For instance, crystal structures of membrane proteins such as the potassium channel[4], bacteriorhodopsin[27], maltoporin[28] 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[23,29,30]. Furthermore, for synthetic transmembrane peptides, tryptophan has been found to be an efficient anchor at the membrane interface[31] and defines the hydrophobic length of transmembrane helices[32]. 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[33,34].

The exact location and orientation of tryptophan residues at the membrane interface is 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[35–38]. 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[35]. The tryptophan-rich aromatic belt at the membrane interface in transmembrane helices are thought to stabilize the helix with respect to the membrane environment[29]. The tryptophan residue has a large indole side chain that consists of two fused aromatic rings. In fact, the tryptophan side chain has the largest volume of all the amino acid side chains[39], with a volume of 228 Å^3 which is comparable to the volume of a phosphatidylcholine headgroup[40], i.e. 319 Å^3. In molecular terms, trypto-

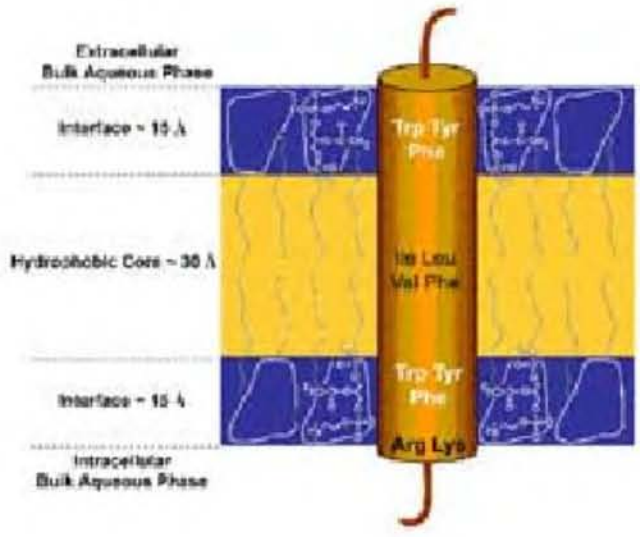

Figure 1. A schematic representation of the membrane bilayer showing the preferred locations of various amino acids present in a transmembrane domain of a membrane protein. The membrane lipids shown have two hydrophobic tails with a phosphatidylcholine (PC) headgroup. It is worth noting that the fluorescent tryptophan residues are localized in the membrane interface, a region characterized by unique organization, dynamics, hydration and functionality. See text for other details. Adapted and modified from ref. 47.

phan is a unique amino acid since it is capable of both hydrophobic and polar interactions. In fact, the hydrophobicity of tryptophan, measured by partitioning into bulk solvents, has previously been shown to be dependent on the scale chosen[41]. Tryptophan ranks as one of the most hydrophobic amino acids on the basis of its partitioning into polar solvents such as octanol[42] while scales based on partitioning into nonpolar solvents like cyclohexane[43] rank it as only intermediate in hydrophobicity. This ambiguity results from the fact that while tryptophan has the polar –NH group which is capable of forming hydrogen bonds, it also has the largest nonpolar accessible surface area among the naturally occuring amino acids[44]. Wimley and White[45] have shown from partitioning of model peptides to membrane interfaces that the experimentally determined interfacial hydrophobicity of tryptophan is highest among the naturally occurring amino acid residues thus accounting for its specific interfacial localization in membrane-bound peptides and proteins. Due to its aromaticity, the tryptophan residue is capable of π–π interactions and weakly polar interactions[46]. 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[34]. The amphipathic character of tryptophan also explains its interfacial localization in membranes due to its tendency to be solubilized in this region of the membrane, besides favourable electrostatic interactions and hydrogen bonding.

Application of fluorescence quenching to membrane proteins and peptides: penetration depths of membrane-bound residues

Fluorescence quenching is operationally defined as a reduction in the measured fluorescence intensity when a fluorophore interacts with another molecule or group, called the quencher. After absorption of a photon, and before emission of radiation, a fluorescent molecule remains in its excited state for a short period of time, usually referred to as the excited state lifetime which is typically in nsecs. If there is an interaction of a fluorophore in the excited state with a quencher, the excited fluorophore may be deactivated before emission of light can take place. The magnitude of quenching depends on the competition between the fluorescence process, the quenching process and other processes that lead to the deactivation of the excited state and is determined by their relative rates. The magnitude of quenching also depends on the concentration of the quencher which is related to the number of quencher molecules in close proximity to the fluorophore.

Depending on the degree of intermolecular motion during the lifetime of the excited state, there could be two major quenching mechanisms, static and dynamic[13]. Static quenching occurs when the distance between the fluorophore and quencher does not change during the lifetime of the excited state of the fluorophore. This is the case for quenching occurring in a solid, or in a frozen or extremely viscous solution, or in a bound 'dark' ground state complex of fluorophore and quencher. Fluorescence quenching occurring in membranes is predominantly static in nature due to slow lateral diffusion ($D = 10^{-8}$–10^{-12} cm^2 s^{-1}) of membrane components. In non-viscous solutions, on the other hand, quenching is largely dynamic because fluorophore-quencher distances change rapidly, i.e. there is relative motion in the nsec time scale. In such cases, quenching interactions occur during periods of close approach of fluorophore and quencher. A special case of dynamic quenching occurs when the range of quenching interactions is sufficiently small so that only collisions between fluorophore and quencher result in quenching of fluorescence. This is called collisional quenching. The rate for such quenching processes is then limited by diffusion, and in cases where quenching is efficient, this rate is the diffusion-controlled collision rate.

Since the extent of fluorescence quenching depends on the proximity (accessibility) of the fluorophore to the quencher, it has been very well utilized to explore the topology (surface or buried) of tryptophan residues in soluble proteins and peptides[48]. The major application of fluorescence quenching in case of membrane proteins and peptides has been to analyse penetration depths of membrane-bound proteins and peptides[13,49]. Membrane penetration depth is an important parameter in the study of membrane structure and organization. The depth of a group within a bilayer provides important information regarding membrane structure including the details of the topography, orientation and folding of membrane-bound proteins and peptides. In a typical quenching experiment using model membranes, a series of molecules labeled with quenchers that occupy different depths in the bilayer are incorporated into the membrane which also contains the fluorophore of interest. The quenchers are often fatty acids or phospholipids with the quencher (spin label groups or heavy atoms such as bromine) covalently attached to the polar headgroup or to a specific fatty acyl carbon atom. This mode of attachment gives the quencher a relatively defined depth provided it does not loop back. In general, phospholipids labeled with quencher groups serve as better probes for such depth studies than quencher-labeled fatty acids for a number of reasons[13]. However, for studies involving native membranes, labeled fatty acids are preferred due to the relative ease of incorporation.

The quencher groups commonly used are dibromo or nitroxide derivatives. The quenching interactions for membrane-bound fluorophores and quenchers are predominatly static in nature with a typical quenching range of 8–12 Å[13,50,51]. The amount of quenching is determined from the ratio of fluorescence in a sample containing the quencher (defined as F) to that in a similar sample in which the quencher is omitted (defined as F_0). The pattern of variation of F/F_0 as a function of the depth of the

quencher is utilized to calculate the depth of the fluorophore. The most popular method of depth analysis is the parallax approach[50] which involves determination of the parallax in the apparent location of fluorophores detected when quenching by phospholipids labeled with quenchers at two different depths is compared. By use of relatively simple algebraic expressions, the method allows calculation of depth in angstroms. This method is relatively simple, yet has proved very useful in a number of cases. For example, the parallax analysis has been used to explore depths of penetration of tryptophan residues and other extrinsic fluorophores in the nicotinic acetylcholine receptor[52], the hemolytic peptide melittin[53], cholesterol oxidase[54], the plant toxin ricin[55], the calcium-dependent membrane binding protein annexins[56], ion channel[57] and fusogenic peptides[58], signal sequence peptides[59], colicin[60], and translocation proteins[61]. Another approach for determining membrane penetration depths utilizing fluorescence quenching data is the distribution analysis[62]. This method uses the Gaussian function to fit the fluorescence quenching profile. This method has been applied to probe depth of penetration of tryptophan residues in Omp A protein[63]. A recent review has highlighted the salient features of both these methods[49]. Very recently, a novel approach has been developed in which the depth of tryptophan residues in membrane embedded peptides are determined by analysis of fluorescence quenching obtained with two quenchers which are not located at fixed depths in the membrane[64].

Site-directed fluorescence labeling approach

The analysis of fluorescence from multitryptophan 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[17]. A novel approach that overcomes the problems associated with proteins containing multiple tryptophans is known as site-directed fluorescence labeling (SDFL)[65,66]. This approach involves covalent attachment of an extrinsic fluorophore to a single site on the target protein. This is accomplished by reacting the fluorophore with the sulfhydryl group in a cysteine residue. The choice of cysteine is due to the fact that in general there is a low abundance of cysteine residues in proteins and also because the chemical modification is done under conditions which do not perturb the structure and function of the protein. In addition, cysteine residues offer ample chemical reactivity for efficient attachment of extrinsic fluorophores such as NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl). In cases where a native protein contains cysteine residues, conventional site-directed mutagenesis approach is utilized to generate a protein containing a single cysteine residue. In this way, one can locate an appropriate fluorescent probe in almost any position of the protein. This approach, therefore, allows the investigator the choice of exploring the environment around every residue in a protein using a variety of fluorescence approaches.

An important application of the SDFL approach is to monitor the insertion or translocation of a soluble protein into membranes. This is based on the fact that SDFL when performed with a polarity-sensitive probe would be able to pick up differences in the environment around the added fluorophore by differences in polarity-dependent fluorescence parameters such as emission intensity and lifetime. For many fluorophores, the emission intensity and fluorescence lifetime show an increase when the fluorophore moves from an aqueous to a non-polar environment. This is also accompanied by a blue shift of the fluorescence emission maximum. A fluorophore which fulfils these criteria rather well is the NBD group. The NBD moiety possesses some of the most desirable properties to serve as an excellent probe for spectroscopic and microscopic applications[67,68]. It is very weakly fluorescent in water. Upon transfer to a hydrophobic medium, it fluoresces brightly in the visible range and exhibits a high degree of environmental sensitivity[69–72]. In addition, fluorescence lifetime of the NBD group is extremely sensitive to the environmental polarity[70,73,74]. The environmental sensitivity of NBD fluorescence is useful in monitoring organization of membrane proteins using the SDFL approach. It has earlier been shown, using solvatochromic and quantum chemical approaches, that the dipole moment of the NBD group changes by ~4D upon excitation[72], an important criterion for a fluorophore to exhibit sensitivity to its environment. For this reason, studies using the SDFL approach have often used the NBD group as the fluorophore of choice. These studies include cotranslational protein translocation and integration at the endoplasmic reticulum (ER) membrane[75–77], and of insertion of pore-forming toxins into membranes[73,78].

Wavelength-selective fluorescence approach

Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system[12,14,47]. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES)[12,14,47,79]. This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises from slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it be-

comes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases will come from the otherwise 'optically silent' water molecules. This makes REES and related techniques extremely useful since hydration plays a crucial modulatory role in a large number of important cellular events, including lipid–protein interactions and ion transport[80,81].

The interfacial region in membranes, characterized by unique motional and dielectric characteristics, represents an appropriate environment for displaying wavelength-selective fluorescence effects. Since the tryptophan residues of membrane proteins are often localized in the interfacial region of membranes (as discussed above), the study of membrane peptides and proteins by the wavelength-selective fluorescence approach utilizing their intrinsic tryptophan fluorescence has become popular. Thus, the environment of the interfacial tryptophan residues in the hemolytic peptide melittin[53,82], the ion channel peptide gramicidin[83], the pore-forming protein α-toxin[84], the synthetic peptides corresponding to a fragment of the ectodomain of the HIV-1 gp41 protein[85], ω-loop region of the human prothrombin γ-carboxyglutamic acid domain[86], γM4 transmembrane domain of the nicotinic acetylcholine receptor[87], and the colicin E1 channel peptide[88] has been monitored using the wavelength-selective fluorescence approach. In addition, wavelength-selective fluorescence approach has also been applied to monitor the environment of the interfacially localized NBD group in membrane-bound fragment of apolipoprotein C-II labeled with NBD group[89].

Conclusion and future perspectives

Monitoring the structure, organization and dynamics of membrane proteins and peptides utilizing fluorescence spectroscopic approaches represent a convenient and sensitive tool with suitable time resolution and minimum perturbation. Against the backdrop of continuing difficulty in successful crystallization of membrane proteins and the subsequent absence of a detailed and exhaustive membrane protein database, fluorescence-based approaches have become increasingly useful. A particular advantage of approaches based on fluorescence spectroscopy is the multiplicity of measurable parameters which complement each other in terms of their information content. Moreover, in cases where an external fluorophore is used, one has a choice of the fluorescent label to be used, and therefore, specific probes with appropriate characteristics can be designed for specific applications. The lack of precise crystallographic resolution is often compensated by the dynamic nature of the information obtained when fluorescence-based approaches are used. In addition, the recent advances in molecular biological techniques in which intrinsic or extrinsic fluorophores of choice can be incorporated in a site-specific manner makes fluorescence approaches very useful[16]. Since a majority of cellular functions are mediated through membrane proteins, which also play a crucial role in pathogenicity[90], information obtained using fluorescence spectroscopy of membrane proteins could prove vital for a better understanding of cellular structure and function in health and disease. This should be reflected in an increasing number of future applications of this approach in investigations of membrane protein structure and function.

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