

REVIEW ARTICLE

# Pore-Forming Protein Structure Analysis in Membranes Using Multiple Independent Fluorescence Techniques

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## Abstract

A large number of transmembrane proteins form aqueous pores or channels in the phospholipid bilayer, but the structural bases of pore formation and assembly have been determined experimentally for only a few of the proteins and protein complexes. The polypeptide segments that form the transmembrane pore and the secondary structure that creates the aqueous-lipid interface can be identified using multiple independent fluorescence techniques (MIFT). The information obtained from several different, but complementary, fluorescence analyses, including measurements of emission intensity, fluorescence lifetime, accessibility to aqueous and to lipophilic quenching agents, and fluorescence resonance energy transfer (FRET) can be combined to characterize the nature of the protein-membrane interaction directly and unambiguously. The assembly pathway can also be determined by measuring the kinetics of the spectral changes that occur upon pore formation. The MIFT approach therefore allows one to obtain structural information that cannot be obtained easily using alternative techniques such as crystallography. This review briefly outlines how MIFT can reveal the identity, location, conformation, and topography of the polypeptide sequences that interact with the membrane.

**Index Entries:** Fluorescence; membrane protein structure; pore-forming proteins; cytolytic toxins; NBD; fluorescence lifetime; fluorescence quenching.

## INTRODUCTION

Most membrane-associated proteins span the bilayer, and many transmembrane (TM)

proteins are involved in the formation of aqueous channels or pores through the membrane. This class of proteins includes polypeptides with very diverse functions, ranging from the porins that passively transport small molecules and ions (1) to the translocon in the endoplasmic reticulum (ER) membrane (2) and the translocases in the mitochondrial membranes

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(3) that transport proteins to the cytolytic bacterial toxins that create pores of different sizes in cellular membranes, some large enough to pass even fully assembled ribosomes (4,5). Although one might expect that these functionally diverse proteins would use the same or similar structural motifs to create an aqueous hole in the nonpolar interior of the bilayer, this does not turn out to be the case. For example, some toxins create pores by the insertion of amphipathic  $\alpha$ -helices into the membrane (e.g., ref. 6), whereas other toxins form pores by the assembly of  $\beta$ -barrels that span the bilayer (e.g., ref. 5). Thus, not surprisingly, one size does not fit all.

Knowledge of the nature of the protein–lipid interactions is essential for achieving an understanding of mechanisms involved in pore assembly and function. For example, nascent membrane proteins are inserted cotranslationally into the bilayer at the ER translocon, a process that requires the translocon proteins to open laterally as a TM domain of the nascent protein moves from the aqueous pore into the hydrophobic interior of the bilayer (2). This unique function clearly requires structural features in the translocon proteins that are not required by other pore-forming proteins. The reversible lateral opening of the translocon pore, as well as its variation in pore diameter (7,8), would appear to rule out pore formation by a  $\beta$ -barrel. Yet, the translocase that facilitates protein translocation across the outer mitochondrial membranes is thought to contain a pore created by a  $\beta$ -barrel (9). Clearly, the structural basis of pore formation must be determined experimentally for each protein or protein assembly.

How does one obtain information about the nature of the TM structure of a protein inserted into a membrane? In particular, how does one identify the polypeptide segments involved in forming the aqueous–lipid interface that defines the pore? Only a few high-resolution structures of biologically relevant membrane proteins have been determined by X-ray crystallography because of the lack of suitable crystals (10). Even fewer structures have been solved by nuclear

magnetic resonance (NMR) spectroscopy because of limitations on the size of the protein that can be analyzed by this technique (11). Such experimental approaches are therefore not the answer. Instead, most researchers have relied on various protein sequence analysis programs to provide an indication of the location and conformation of the TM sequences in a protein (12). Although this approach is usually correct for completely nonpolar TM  $\alpha$ -helices, it is less successful when a TM segment is in an amphipathic  $\beta$ -conformation or when polar residues are present in an amphipathic  $\alpha$ -helix, the situations one expects to encounter during analyses of pore-forming proteins.

It is therefore desirable to establish methods for obtaining this structural information that are more accessible to investigators than X-ray crystallography and NMR spectroscopy. A number of biophysical and chemical methods have been employed successfully by researchers to characterize structural aspects of protein–membrane interactions. These techniques include fluorescence spectroscopy (e.g., refs. 2 and 5), electron paramagnetic resonance (e.g., ref. 13), and infrared spectroscopy (e.g., ref. 14). Here, we shall focus on fluorescence spectroscopy.

## FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy can provide valuable information about the identity, location, conformation, and topography of the polypeptide sequences that interact with a membrane and thereby provide insight into the mechanisms of protein insertion into and interaction with membranes. Which region of the protein interacts with the membrane bilayer? What secondary structure is adopted by the polypeptide during membrane binding or insertion? By what mechanism does the protein interact with the membrane? These and many other questions can be answered by combining the appropriate fluorescence techniques. We will discuss a general approach to examine protein–membrane structure using fluorescence spectroscopy. Although most of our examples will

involve a particular bacterial pore-forming toxin, perfringolysin O (PFO), the approach can be easily adapted to analyze a wide variety of proteins that associate with or insert into a membrane bilayer.

The primary reason for using fluorescence spectroscopy is its sensitivity, both in terms of the wide range of information one can extract from an analysis of the fluorescence signal and also in terms of the small amount of material required to obtain a measurable signal (less than a picomole at nanomolar concentrations). The technique is nondestructive, so one can monitor the kinetics of processes that elicit spectral changes, as well as examine the equilibrium states of samples. With the best instruments, materials, and choice of fluorophore, background signal and light scattering are insignificant. In addition, one can focus on a particular feature or property of the fluorophore environment by selecting the appropriate fluorescent dye or probe. Finally, one can easily introduce a single extrinsic probe into any specific site in a polypeptide using site-directed fluorescence labeling (SDFL, *see* following section); hence, one can, in principle, examine every residue in the polypeptide. Most important, the fluorescence approach provides a means to characterize the surroundings of any protein residue before, during, and after the polypeptide interacts with the membrane bilayer. Therefore, this method provides important information that is not available using crystallography, NMR, or other techniques.

## INTRINSIC AND EXTRINSIC PROBES

Both intrinsic and extrinsic fluorophores have been used as reporters of protein–membrane interactions. In proteins, the dominant intrinsic fluorophore is the indole group of tryptophan. Trp residues have a maximum absorbance near 280 nm, and the maximum emission intensity ranges from 310 to 350 nm, depending on solvent polarity. The emission maximum is blue-shifted if the Trp is in a hydrophobic environment and is red-shifted

when the Trp is exposed to an aqueous environment [(15) and references therein].

Intrinsic fluorophores are often effective reporters of protein conformational changes (e.g., ref. 16) and can be especially valuable if a single Trp residue is located in a region of protein that interacts with the membrane (17). In this ideal case, an increase in Trp emission intensity is expected if the Trp is exposed to water in the soluble protein and buried in the bilayer when membrane bound, and the resulting signal change can be used to monitor the protein–membrane interaction. However, many proteins contain multiple Trp residues, and the assignment of a change in fluorescence signal to a specific membrane-interacting region of the protein is then more difficult. For example, the cytolytic toxins leukocidin F, anthrax protective antigen, PFO, and *Staphylococcus aureus*  $\alpha$ -hemolysin contain 6–8 Trp residues and proaerolysin has 18 Trp residues. For such proteins, the identification of a Trp(s) responsible for a particular spectral change is usually accomplished by replacing each Trp in turn with a Phe to determine which portion of the protein is interacting with the membrane (e.g., ref. 18) or by creating single-Trp variants of the protein (e.g., ref. 17).

An alternative approach that overcomes the problems associated with proteins containing multiple intrinsic fluorophores is known as SDFL. This approach involves the covalent attachment of an extrinsic fluorophore to a single site on the target protein. This is often accomplished by reacting the fluorophore with the sulfhydryl group in a Cys, both because Cys has a low abundance in proteins and because the modification chemistry proceeds under conditions that do not compromise protein structure or function. For those cases when a native protein contains Cys residues, standard site-specific mutagenesis techniques are used to create a single-Cys protein. For example, a naturally occurring Cys could be replaced by an appropriate amino acid in order to allow specific labeling of the protein (e.g., ref. 19). Of course, a Cys-less protein is the optimal substrate for the SDFL approach, but

the presence of Cys residues in a disulfide bond (20,21) or an unreactive Cys residue buried in the protein and not exposed to the labeling reagent (e.g., ref. 22) will not interfere with the labeling reaction. In this way, one can locate an appropriate fluorophore at almost any position in the polypeptide.

Although the replacement of a natural amino acid by a Cys residue usually does not affect the structure or function of the protein, such a modification sometimes does change the properties of the mutant protein (activity, stability, folding, etc.). Therefore, it is important to evaluate for each mutant whether the introduced modification alters the protein significantly. For proteins with a measurable functional activity, the activity of a mutant should be compared to that of the wild-type protein (e.g., ref. 19). For other proteins, structure-based assays must be used. For example, comparing the stability of the mutant (e.g., using thermal unfolding) to that of the wild-type protein may reveal modification-dependent changes in protein folding or assembly (e.g., ref. 23). It is also important to note that the production of an unstable or inactive mutant provides valuable information even though it is useless for spectroscopic measurements. In such a case, the mutated amino acid must be essential for some aspect of protein structure or function, such as, among other things, the correct folding of the polypeptide, a role in the active site or ligand-binding site of the protein, and/or its interaction with a membrane.

## MEMBRANES

To properly analyze protein-membrane interactions, it is obviously important to choose an appropriate membrane system. Although natural membranes have been used successfully in studies involving extrinsic fluorophores (e.g., refs. 19,24, and 25), a membrane sample that provides a minimal fluorescence background and the flexibility to modify its chemical composition is desirable.

Large unilamellar vesicles (0.1–0.2  $\mu\text{m}$  in diameter) provide an excellent alternative to

natural membranes in many fluorescence spectroscopic analyses. Such liposomes can be prepared easily, provide minimal fluorescent backgrounds, and allow variation of the lipid composition of the membrane. In addition, specific probes can be incorporated into the bilayer as fluorescence quenchers (e.g., ref. 19) or as donors or acceptors in fluorescence resonance energy transfer (FRET) measurements (e.g., refs. 26, and 27). Also, fluorescent markers of different sizes can be encapsulated into the liposomes to analyze various aspects of pore formation (e.g., ref. 28). Liposomes prepared by extrusion techniques (29) provide a relatively homogenous sample in size and lipid composition (30).

## SELECTING THE APPROPRIATE EXTRINSIC FLUOROPHORE

The ideal extrinsic fluorescent probe for spectroscopic studies will not influence the conformation or location of the polypeptide to which the dye is covalently attached. In addition, the probe will be soluble in both aqueous and nonaqueous milieus so that it can serve as a stable reporter in both environments. This flexibility will also minimize the possibility that the properties of the probe will drive an alteration in protein structure or folding.

Because protein association with or insertion into membranes usually involves a transition from a water-soluble state to a membrane-associated state, SDFL with a water-sensitive fluorescent probe can be used to identify the membrane-interacting regions of the protein. For most such dyes, the emission intensity and fluorescence lifetime increase when the fluorophore moves from an aqueous to a nonaqueous milieu, and the maximum emission wavelength is shifted to the blue. Naphthalene derivatives like acrylodan (31,32) and badan have been used as polarity-sensitive probes in protein-membrane structural studies (e.g., refs. 33 and 34) (Fig. 1). However, the poor solubility of these compounds in water may cause the dye to bury itself in the bilayer (or in a

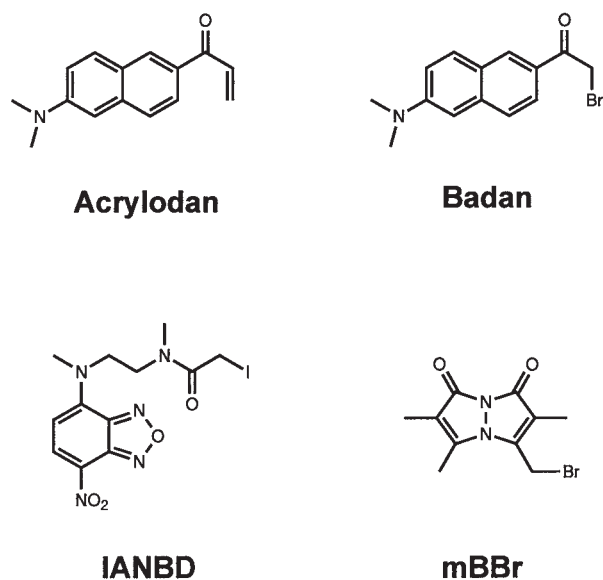


Fig. 1. Structures of a few water-sensitive fluorescent probes employed in protein-membrane analyses: acrylodan (6-acryloyl-2-dimethylaminonaphthalene), badan (6-bromoacetyl-2-dimethylaminonaphthalene), IANBD [N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethyl]diethylamine, and mBBR (monobromobimane).

hydrophobic pocket of the protein) even when the side chain it replaced would have been stably exposed to the aqueous environment.

Alternatively, polarity-sensitive fluorophores that are more water soluble, such as NBD (7-nitrobenz-2-oxa-1,3-diazole) (35,36) and monobromobimane (23,37,38) have been used successfully in SDFL (Fig. 1). NBD fulfills the above-mentioned requirements because NBD has a relatively small size for a dye, it is uncharged, and its N and O atoms give the dye sufficient polar character to be soluble in an aqueous environment. Equally important, the emission intensity and fluorescence lifetime of NBD increase substantially upon moving from an aqueous solvent to the nonpolar core of the bilayer (19,36). NBD has been used as a reporter group in many studies, including investigations of cotranslational protein translocation

and integration at the ER membrane (e.g., refs. 2,24, and 25) and of toxin insertion into bilayers (e.g., refs. 5,28, and 39).

## MULTIPLE INDEPENDENT FLUORESCENCE TECHNIQUES

To illustrate how one can achieve an unambiguous determination of the identity and conformation of a TM segment of a membrane-interacting protein, we will describe how the SDFL approach was used to characterize PFO. This approach allowed us to identify TM domains of PFO and, by using multiple independent fluorescence techniques (MIFT), to analyze not only the secondary structure adopted by these TM domains after pore formation but also the topography of this polypeptide in its membrane-inserted form.

### Searching for a Membrane-Interacting Domain

Perfringolysin O is one of many pore-forming toxins that are secreted from bacterial cells, fold into a stable water-soluble intermediate state, and ultimately convert spontaneously into an oligomeric membrane-inserted conformation that punctures the membrane of the target cell (Fig. 2) (4,5). In order to elucidate the mechanism of pore formation for a particular toxin, it is important to identify which region(s) of the toxin polypeptide interacts with and/or inserts into the membrane (colored regions in Fig. 2).

To identify a membrane-interacting domain of a protein using SDFL, the basic experimental strategy is to position a single water-sensitive fluorescent probe at many different places in the target protein and then compare the fluorescence signal of the probe in each labeled mutant before and after the protein contacts the membrane. A potential membrane-interacting residue would be revealed each time that a large change in the fluorescence signal of the dye is detected upon pore formation. For example, no significant fluorescence intensity

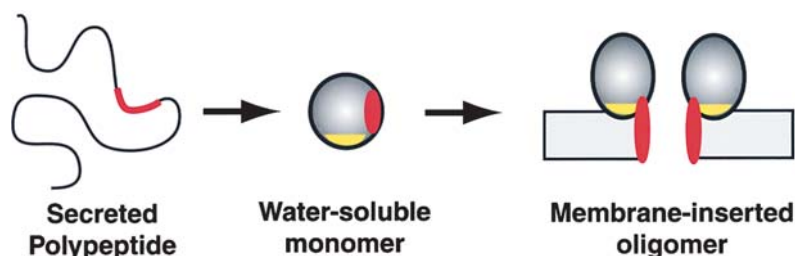


Fig. 2. Schematic model of the structural transitions of a pore-forming toxin. The unfolded polypeptide is secreted from the bacterial cell and is then folded into a water-soluble protein. After recognizing a target membrane, the toxin binds and inserts into the bilayer, often in multimeric complexes containing 7–50 subunits. The regions of the toxin that contact the membrane are colored in yellow (membrane-interacting domain) and red (membrane-inserted domain).

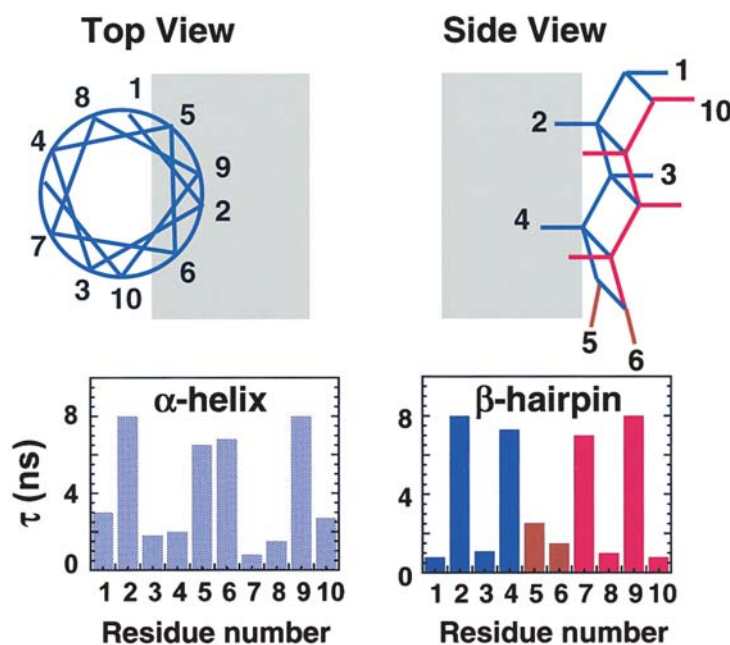


Fig. 3. Secondary structure analysis of a TM polypeptide using fluorescence spectroscopy. The upper panel shows the two different secondary structures that a TM polypeptide can adopt after insertion into the membrane: an  $\alpha$ -helix (left, top view) or a  $\beta$ -hairpin (right, lateral view). The lower panel shows the expected patterns for the NBD fluorescence lifetime analysis of mutants labeled at successive residues in the TM polypeptide of the toxin. A long fluorescence lifetime ( $\tau$ ) indicates exposure to the membrane bilayer, whereas a short lifetime indicates exposure to water (i.e., facing the pore). The numbers indicate the consecutive residues in the primary structure of this arbitrary TM segment. For clarity, the two strands of the  $\beta$ -hairpin are colored red and blue and the turn is colored brown.

increase is expected for an NBD dye attached to a residue that is located on the water-exposed surface of the toxin (colored gray in Fig. 2) both before and after pore formation. However, when a water-sensitive NBD dye is attached to a residue of the toxin that contacts the hydrophobic interior of the membrane (colored yellow and red in Fig. 2) after pore formation, a large increase in NBD fluorescence intensity will be observed. Such a spectral change is therefore indicative of a membrane-interacting region of the toxin. The analysis can then be extended to the adjacent amino acids to evaluate further the characteristics of this membrane-interacting domain (How large is the domain? How deep does it insert into the membrane?). Because the TM sequences used by proteins to create a pore in the membrane are amphipathic in nature, important structural information can also be obtained from the analysis of the fluorescent-labeled mutants in this region.

### Secondary Structure Analysis

Two different secondary structures are expected for the regions of a polypeptide that span the membrane and form a boundary between the lipid bilayer and an aqueous pore: an amphipathic  $\beta$ -sheet conformation or an amphipathic  $\alpha$ -helical conformation (Fig. 3). For a  $\beta$ -sheet conformation, the environment of residues along the membrane-bound polypeptide alternates between aqueous (facing the pore) and nonpolar (facing the core of the bilayer). Alternatively, for an amphipathic  $\alpha$ -helix, the residues facing the aqueous pore or the nonpolar bilayer would conform to that expected from a helical wheel analysis of the location of the residues in the  $\alpha$ -helix.

The pattern of hydrophobic environments (alternating or helical wheel) can often be inferred from changes in fluorophore intensity that result from membrane insertion of the labeled mutants. For example, NBD probes placed at successive sites in a stretch of polypeptide may show a pattern of spectral changes consistent with a  $\beta$ -sheet because the NBD located at every second residue exhibits a large increase in

fluorescence intensity. However, it is important to recognize that this approach, comparing intensity after membrane insertion ( $F$ ) to intensity prior to insertion ( $F_0$ ), can be misleading because the final  $F/F_0$  ratio obviously depends on the initial  $F_0$  value. For example, a probe initially buried inside a folded PFO monomer will show little or no intensity change if it ends up in the similarly nonpolar interior of the bilayer. In such a case, a probe could insert into the bilayer without experiencing a large increase in emission intensity ( $F/F_0$  would be close to 1.0). Moreover, a fluorescence intensity measurement does not reveal the extent of fluorophore heterogeneity in the sample. For example, a twofold increase in observed emission intensity could result from 100% of the probes doubling their intensity or from 50% of the probes quadrupling their intensities while 50% experienced no change in intensity. Thus, one needs to complement intensity measurements with measurements that can assess sample homogeneity. To determine unambiguously the environment and homogeneity of NBD probes in a sample, time-resolved fluorescence techniques can be used to measure the fluorescence lifetime of every probe in the sample (e.g., refs. 19 and 24).

### Lifetime Measurements and Probe Environment

Fluorescence lifetime measurements are particularly valuable because they reveal directly the environment of a dye and, in particular, its exposure to water (19,36). A probe in a particular environment has a specific fluorescence lifetime (e.g., NBD has an emission lifetime of approx 1 ns in an aqueous medium and 7–8 ns when in the nonpolar core of the bilayer), so the detection of multiple lifetimes in a sample demonstrates directly that the probes are in multiple distinguishable environments. By determining the fraction of dyes in each environment, one can quantify the extent of sample heterogeneity. Hence, one can ascertain the homogeneity of the sample both before and after membrane insertion and can determine whether each protein in a sample undergoes

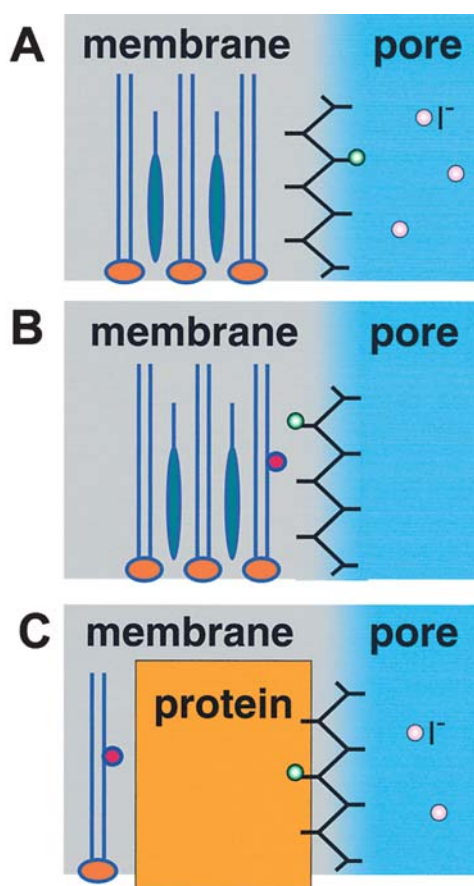


Fig. 4. Determination of probe location by its accessibility to quenchers. (A) Only a probe (colored green) exposed to the aqueous medium is quenched by hydrophilic quenchers such as iodide ions (pink). (B) Only a probe facing the membrane bilayer is quenched by membrane-restricted quenchers (e.g., doxyl-phosphatidylcholines) (colored red). (C) Probes buried in a protein (orange) are not quenched by membrane-restricted quenchers.

the same transition during membrane insertion. Such quantitative data cannot be elicited solely from measurements of changes in emission wavelength or intensity before and after membrane insertion.

Moreover, when examining a pore-forming protein by SDFL, the pattern obtained for the fluorescence lifetimes of different mutants after membrane insertion provides direct informa-

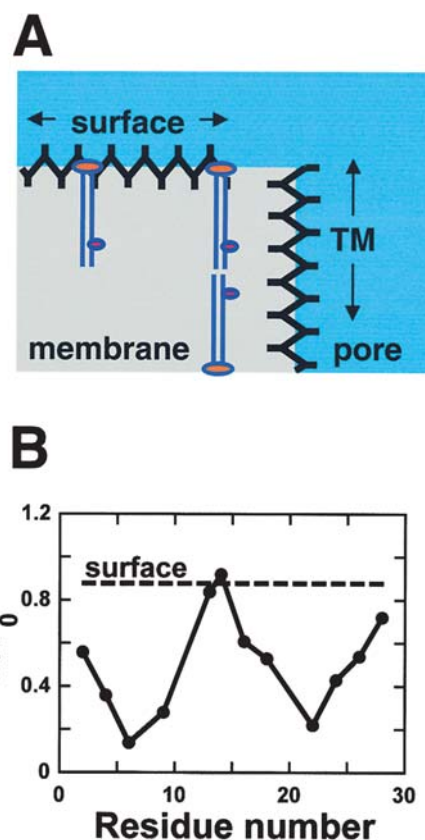


Fig. 5. The topography of a membrane-interacting polypeptide. (A) Two possible orientations of an amphipathic polypeptide that interacts with the membrane bilayer: lying on the surface of the membrane or spanning the membrane (TM). (B) Quenching pattern obtained for a TM  $\beta$ -hairpin that spans the membrane (solid line) and the pattern of quenching efficiency expected for a polypeptide that lies on the surface of the membrane (dashed line). Experimental details are described in ref. 39.

tion about the secondary structure adopted by a TM sequence (Fig. 3) (19,39).

### *Distinguishing Nonpolar Environments in Proteins and Membranes*

Fluorescence lifetime analysis cannot by itself distinguish between probes in environments

that are different but have very similar fluorescence lifetimes, such as a probe located in the hydrophobic core of a membrane and a probe in the hydrophobic core of a protein (compare panels B and C in Fig. 4). Thus, an additional independent experimental approach is necessary to determine probe location unambiguously. One such approach is collisional quenching, a technique that relies on the ability of some molecules and ions to dissipate the excited-state energy of an excited fluorescent dye upon colliding with it. Such collisions therefore reduce or quench the fluorescence emission of the sample. Because sample emission is decreased only when a dye collides with a quencher molecule or ion, this technique constitutes a direct measure of the accessibility of the probe to the quencher species (Fig. 4).

The extent of probe exposure to a quencher can be estimated by the magnitude of the quencher-dependent reduction in fluorescence emission, if any. The location of probes in a sample can therefore be determined by using collisional quenchers that are themselves restricted to certain locations within the sample (e.g., refs. 19, 24, 25, and 39). For example, only a probe attached to a residue that faces the aqueous medium in either a soluble or membrane-inserted pore-forming toxin will be quenched by hydrophilic quenchers such as iodide ions (Fig. 4A). Similarly, a quencher restricted to the nonpolar interior of the membrane (e.g., a nitroxide moiety covalently attached to an acyl chain of phosphatidylcholine) will quench a probe that faces or is embedded in the lipid bilayer (Fig. 4B), but it will not quench a probe that is located in the nonpolar interior of a protein domain (Fig. 4C). To identify probes exposed to the interior of a membrane, 7-doxyl-labeled phosphatidylcholine (7-doxyl-PC) is perhaps the best lipophilic quenching reagent because the nitroxide moiety that serves as the quencher is positioned approximately in the middle of each leaflet of the bilayer. The dynamic motion of the flexible acyl chain will allow the nitroxide to contact and quench each NBD probe facing the bilayer, although to different extents.

Thus, NBD attached to residues that face the aqueous milieu will be quenched only by

hydrophilic quenchers, whereas those that are buried in the interior of the membrane will be quenched only by doxyl-labeled phospholipids. Those probes located inside a protein domain will not be quenched by either iodide ions or doxyl-PC. In the case of PFO, this quenching approach allowed us to identify unambiguously NBD probes that were buried in the bilayer because their fluorescence lifetimes increased to 7–8 ns and their emission was quenched by 7-doxyl-PC (19,39).

### ***Does the Amphipathic Polypeptide Span the Membrane?***

The above spectral analysis can identify residues exposed to the membrane interior, but it cannot distinguish between a polypeptide that lies on the membrane surface (Fig. 5A) and one that spans the bilayer (TM in Fig. 5A). To distinguish between these two possibilities, one can use a lipophilic collisional quencher, 12-doxyl-PC, whose nitroxide moiety is located near the center of the bilayer (39). Although the nitroxide attached to a flexible acyl chain in a fluid bilayer can dynamically reach and quench probes attached to most sites on a TM polypeptide, NBD dyes will be quenched most efficiently when they are located at the same level in the bilayer as the average position of the nitroxide (40). Thus, if the amphipathic polypeptide spans the bilayer, one would expect to see different extents of quenching by 12-doxyl-PC for NBD dyes positioned at different sites along the polypeptide and, hence, at different locations within the bilayer (Fig. 5B). In contrast, if the amphipathic polypeptide is lying along the membrane surface, then the NBD dyes replacing each of the residues in the polypeptide that face the bilayer would all be located at the same depth within the nonpolar core and, hence, would all be quenched to the same extent (or nearly so) by 12-doxyl-PC (dashed line in Fig. 5B). This approach allowed us to demonstrate unambiguously that the two membrane-bound  $\beta$ -hairpins of PFO were oriented so as to span the bilayer, not lie on its surface (39).

### ***Protein–Membrane Topography***

In addition to identifying which residues of a protein comprise the TM segment(s), fluorescence spectroscopy can be used to determine the topography of individual residues and, hence, various domains of a protein relative to the membrane surface. For example, the locations of the active sites of each of the membrane-bound enzymes involved in blood coagulation have been determined by FRET. By covalently attaching a donor dye to an active-site residue and then measuring the efficiency of FRET to acceptor dyes distributed randomly and uniformly at the membrane surface, the active sites of factor Xa (26,41), factor IXa (41), factor VIIa (42), activated protein C (43), meizothrombin (44), and the thrombin-thrombomodulin complex (45) were shown to be located more than 70 Å above the membrane surface. These studies also demonstrated that the height and/or orientation of each active site except one was altered when the enzyme associated on the membrane surface with the cognate nonenzymatic protein cofactor required for activity. The cofactor-dependent change in active-site location therefore constitutes a critical mechanism for physiologically regulating blood clotting (e.g., ref. 27). In addition to providing topographical information, FRET can also be used to characterize the thermodynamics and kinetics of protein binding to a membrane surface (e.g., ref. 46) or to detect protein–protein association on the membrane surface (e.g., ref. 47).

### ***From Structure to Mechanism: Kinetic Analysis***

Each of the above fluorescence measurements can be monitored as a function of time, as can a fluorescence technique that we have not discussed here: fluorescence polarization or anisotropy. One can therefore determine the kinetics of any spectral change and thereby obtain information that may reveal the mechanism by which a particular process is accomplished. For example, by monitoring intrinsic and extrinsic probes located in different domains of PFO, we were able to identify by

kinetics an ordered and sequential interaction of different domains of PFO with the membrane (28). These kinetic data therefore showed which domain of PFO is responsible for recognizing whether the membrane contains sufficient cholesterol to support the binding of PFO.

## **CONCLUSIONS**

Important information about the nature of a protein's interactions with a membrane is available by the application of various fluorescence techniques. By combining the results obtained from multiple independent fluorescence techniques as described here, one can reduce uncertainties in interpretation and unambiguously determine the identity and conformation of the polypeptide stretches that are exposed to the interior of the membrane. The MIFT approach therefore constitutes a more accessible alternative than X-ray crystallography or NMR spectroscopy to characterize protein interactions with membranes. In fact, fluorescence spectroscopy is arguably a better approach for examining protein–membrane interactions because of its ability to examine proteins inserted into or associated with an intact membrane, to examine multicomponent complexes, and to measure the kinetics of any spectral (and hence structural) change.

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