Fluorescence Spectroscopy in Peptide and Protein Analysis

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Fluorescence spectroscopy and its multiple applications to the life sciences have undergone rapid development. This is due to numerous technical advances in both instrumentation and methods of data analysis as well as to a vast proliferation of basic techniques. Applications of fluorescence spectroscopy to protein and peptide analysis are governed by three principal factors: the dynamic nature of the signal, its localized nature, and its redundancy. Although these features can complicate interpretation of the experimental result, they also can be exploited to obtain unique structural and dynamic information. The availability and simplicity of basic data acquisition and analysis are important practical features behind the popularity of fluorescence as compared to other spectroscopic techniques. Yet this simplicity does not appear to compromise its advantages. This article is intended first to provide an overview of the fluorescence phenomenon in proteins, and second to illustrate applications of fluorescence spectroscopy in advanced (but not necessarily high-tech) studies. Three areas of protein studies, namely protein–ligand interactions, protein folding and studies of membrane proteins, have been chosen to demonstrate key advantages of fluorescence spectroscopy: it is sensitive, versatile, and it lends itself readily to fast data acquisition.

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1 INTRODUCTION

Fluorescence spectroscopy and its multiple applications to protein analysis, and to the life sciences in general, have undergone rapid development during the past decade. This progress appears to be driven on two levels. First, numerous technical advances in time resolution, methods of data analysis, and improved instrumentation have enabled researchers to probe the structural and dynamic features of proteins, membranes and nucleic acids, to acquire multidimensional (space–time) microscopic images of the distributions of various molecules in cell cultures, to follow conformational changes of single molecules, etc. These developments, pioneered by a handful of research groups, have spilled over into multiple areas as diverse as basic analytical chemistry and practical clinical applications.

The second, and sometimes under-appreciated, level of development of fluorescence spectroscopy in studies of biological macromolecules involves the proliferation of the basic technique. This progress is driven mainly by researchers whose primary scientific interests lie well outside the field of spectroscopy, and who, while admiring the elegance of the custom-designed multiphoton excitation experimental scheme, might not necessarily want to implement one in their own laboratory. Instead, they will use a commercially available fluorimeter, almost standard equipment these days in many laboratories involved in biochemical and biophysical studies. Nevertheless, their studies have produced not only numerous answers to a variety of important biological problems but have broadened the range of fluorescence techniques as well.

The availability and simplicity of basic data acquisition and analysis are important practical features behind the popularity of fluorescence as compared to other spectroscopic techniques. Yet this simplicity does not appear to compromise its main advantages, one of which, exceptional sensitivity, allows routine detection of fluorescent substances on a subnanomolar scale on the one hand, and provides the ability to collect data in a kinetic regime during fast submillisecond reactions on the other. Applications of fluorescence spectroscopy to protein and peptide analysis are governed by three principal factors: (1) the dynamic nature of the signal, (2) its localized nature, and (3) its redundancy. Fluorescence is a dynamic phenomenon and the

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lifetime of the excited state is sufficient for a variety of chemical and physical reactions to take place prior to emission. The usual nanosecond time-window of fluorescence is normally shorter than that of other dynamic techniques (e.g. nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR)). Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore, which distinguishes it from generalized techniques, such as calorimetry, far-ultraviolet circular dichroism (CD), and infrared (IR) spectroscopy. Also, various possibilities of structural rearrangements in the environment of the fluorophore may lead to a similar fluorescence signal. None of these features taken alone is either beneficial or detrimental. Although they can complicate interpretation of the experimental result, they also can be exploited to obtain unique structural and dynamic information.

Fluorescence spectroscopy and its various applications have been reviewed over the years in several excellent publications, e.g. basic(1) and advanced textbooks,(2,3) monographs(4,5) and specialized editions.(6,7) This article is not intended as a condensed version of the material in those contributions. Instead it focuses on two tasks: first to provide an overview of the fluorescence phenomenon in proteins, and second to illustrate applications of fluorescence spectroscopy in state-of-the-art (but not necessarily high-tech) studies. The goal is to concentrate on fundamental principles and advanced applications.

1.1 What is Measured in a Protein Fluorescence Experiment?

The four principal aspects of the fluorescence phenomenon are: energetics, probability, kinetics and vectoriality. Each can be studied experimentally by measuring the intensity of the emitted light as a function of wavelength, orientation of polarizers, time elapsed since excitation, etc. Therefore the question posed in the title of this section has a simple answer – fluorescence intensity, I. Let us now consider how this basic measurement can be converted into multiple measures characterizing various aspects of fluorescence.

Intensity measured as a function of wavelength (or wavenumber) comprises the fluorescence spectrum. The position of the emission spectrum reflects changes in energetics of the excited and ground state between excitation and emission of a photon. In practice the position of the emission band is expressed as a wavelength of the maximum λ_{max}.

Total intensity of the emission at all wavelengths is related to fluorescence quantum yield, q. Quantum yield is defined as a ratio of the number of emitted quanta to the number of absorbed quanta. It reflects the probabilities of various photochemical and photophysical processes leading to radiational or radiationless deactivation of the excited state.

The time-dependence of fluorescence intensity following excitation in the ensemble of fluorophores is related to the excited state lifetime, τ. This kinetic parameter characterizes the average time a molecule spends in the excited state before emitting the photon:

$$I(t) \sim e^{-t/\tau}$$

More often than not, fluorescence intensity follows a more complex law of decay than simple exponential (see section 2.3). Two experimental schemes allowing determination of the fluorescence lifetime are known as pulse domain and frequency-modulation domain spectroscopy (for more on lifetime measurements see Lakowicz(1,3)).

Anisotropy, r (along with the interchangeably related polarization, P), is a vectorial characteristic reflecting changes in the direction of the emission transition moment with regard to the excitation transition moment. Anisotropy is affected by the relative orientation of the dipoles in the ground and excited states and by changes in the orientation of the excited state with respect to external (laboratory) coordinates due to the molecule's rotation or radiationless energy transfer to another molecule prior to emission. Anisotropy and polarization are defined in Equations (1) and (2) by intensities of vertically ($I_{\parallel}$) and horizontally ($I_{\perp}$) polarized light measured when excitation light is vertically polarized:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1}$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{2}$$

Anisotropy and polarization can be measured in a steady-state and kinetic regime (for more on anisotropy measurements and their applications see Steiner(8)).

The ratio of intensities in the absence, I_0, and in the presence, I, of an external quencher at concentration $[Q]$ is used to determine a Stern–Volmer constant, K_{SV}, as shown in Equation (3):

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \tag{3}$$

If the changes in lifetime follow the changes in intensity, the quenching is called dynamic and the biomolecular quenching rate, k_q, can be determined using Equation (4):

$$\frac{\tau_0}{\tau} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \tag{4}$$

The biomolecular rate gives information on chromophore accessibility to the aqueous phase and on the distribution

of charges surrounding the fluorophore if a charged quencher is used. In the case of static quenching, only the intensity decreases with addition of quencher while the lifetime remains the same (for more on quenching measurements and their applications see Eftink[9]).

Another useful parameter is the efficiency of the long-range energy transfer, E, between an initially excited molecule (donor) and another chromophore (acceptor). Efficiency of this dynamic process can be calculated from either intensity or lifetime of donor alone (I_D, τ_D) and in the presence of acceptor (I_{DA}, τ_{DA}) as shown in Equation (5):

$$E = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (5)$$

Efficiency of transfer is used to estimate the distance between donor and acceptor, R, according to Förster theory, as shown in Equation (6):

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (6)$$

where R_0 is a Förster distance for half-transfer and is a characteristic of a donor–acceptor pair and its environment (for more on energy transfer measurements and their applications see Cheung,[10] Wolber and Hudson,[11] and Wu and Brand[12]).

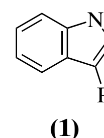
It is important to keep in mind that neither distance, nor quenching rate, nor strictly speaking lifetime are *measured* in a fluorescence experiment. All these important parameters are derived from the basic measurements of intensity, using more or less restrictive assumptions (e.g. absence of nonspecific perturbations after introduction of the acceptor fluorophore, or fluorescence decay being adequately described by a sum of exponential components) and therefore are to some extent model-dependent.

2 PROTEIN FLUORESCENCE

2.1 Spectral Properties of Protein Fluorophores

2.1.1 Tryptophan and its Derivatives

Absorption of tryptophan is due to $\pi \rightarrow \pi^*$ transitions in the indole ring **(1)**. The short wavelength band (220 nm) is due to the ^1B_b transition while the long wavelength band (260–290 nm) consists of the two overlapping transitions ^1L_a and ^1L_b with vectors almost perpendicular to each other. The ^1L_a transition is sensitive to the polarity giving rise to a slight shift in absorbance spectrum. The ^1L_a transition is believed to be the main contributor to the emission.



(1)

In aqueous solution tryptophan displays a wide, structureless fluorescence band with a maximum of about 350 nm and width of about 60 nm. It is worth noting that tryptophan fluorescence spectra measured under the same conditions but using different instruments may differ slightly in position of the maximum (348–353 nm), width and shape because of the differences in spectral calibration. Unfortunately, at present there are no generally accepted standards for the spectral calibration of instruments in the near-ultraviolet region. Because excitation leads to a substantial increase in the dipolar moment (about 4 D), shifts in the emission are much stronger. This shift is caused by the orientational relaxation processes involving the dipole of the chromophore and solvent dipoles. This sensitivity of tryptophan emission to polarity and mobility of environment makes tryptophan fluorescence an important tool in studies of protein structure and dynamics.

The absolute quantum yield of tryptophan in an aqueous environment was reported to be 0.13.[13] In most cases, however, a knowledge of the absolute value is not necessary and relative quantum yield is used instead. Tryptophan fluorescence is susceptible to the quenching by water which occurs with high activation energy (11–12 kcal mol^{-1}).[14,15] This leads to the frequently observed decreases in quantum yield upon denaturation when tryptophan becomes exposed to an aqueous environment. However, there are numerous exceptions to this rule because tryptophan fluorescence can also be quenched in a protein's native state.

Almost all polar protein groups can quench tryptophan fluorescence, to some extent.[14,16–21] Possible nonradiative processes include photoionization, intersystem crossing, exciplex formation, and excited state proton and excited state electron transfer. Aspartic and glutamic acid residues are effective dynamic quenchers in their neutral, but not charged, forms. Lysine and arginine are also dynamic quenchers, but are more effective when charged. Histidine at low pH appears to quench by formation of a stacking complex with the indole ring. The nonprotonated histidine is also able to quench tryptophan fluorescence, although with much-reduced efficiency. Disulfide is one of the strongest quenchers of tryptophan fluorescence, although single cysteine can be an effective quencher too. In addition, amide and peptide groups were shown to act as dynamic quenchers. In heme-containing proteins, long-range energy transfer quenching is important due to the spectral overlap

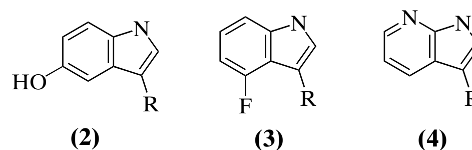
of tryptophan emission and heme absorbance. While such effects complicate the analysis of protein fluorescence, they can also be used to extract valuable structural information. For example, in membrane-bound cytochrome b_5, heme quenching of Trp-109 was used to estimate the distribution of distances between two protein domains.[22]

Tryptophan appears to be uniquely sensitive to quenching by a variety of substances, such as oxygen, iodide, bromide, acrylamide, succinimide, hydrogen peroxide, dichloroacetamide, pyridinium hydrochloride, NO_3^-, Cs^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Mn^{2+}.[14,16,23] This sensitivity to quenchers allows determination of the accessibility of the tryptophan residues in proteins by quenching measurements.

The fluorescence decay of tryptophan does not follow a single exponential. Szabo and Rayner[24] had reported two decay times of 0.5 and 3.1 ns for tryptophan zwitterion in an aqueous environment at room temperature. The short-lived component has a blue-shifted spectrum. The heterogeneity of decay of tryptophan derivatives in fast-relaxing environments is attributed to the side chain rotamers. Decay becomes even more complex when solvent relaxation occurs on a nanosecond timescale (see section 2.3).

The existence of two overlapping transitions and energy transfer occurring predominantly from ^1L_b to ^1L_a complicates the appearance of the excitation polarization spectrum of tryptophan. In their classic work, Valeur and Weber[25] have resolved two transitions in the excitation spectrum of indole and tryptophan in frozen propylene glycol using polarization data. The limiting anisotropy of tryptophan depends on the excitation wavelength and is about 0.3 at 300 nm, and 0.2 at 270 nm. The spectral region in-between has several sharp bands, which are expected to shift depending on the solvent conditions, making the region from 280 to 300 nm difficult to use for polarization measurements.

An important emerging field in protein fluorescence is related to the use of spectrally enhanced protein mutants.[26] This approach takes advantage of tryptophan analogs with different photophysical properties incorporated as intrinsic fluorophores either by chemical synthesis or, biosynthetically, using tryptophan auxotroph *Escherichia coli* strains.[27,28] Three analogs are suggested to be most useful for studies of protein–protein and protein–nucleic acid interactions: 5-hydroxytryptophan **(2)**, 4-fluorotryptophan **(3)** and 7-azatryptophan **(4)**. 5-Hydroxytryptophan absorbance has a long wavelength shoulder going as far as 320 nm, allowing this fluorophore to be selectively excited in the presence of multiple normal tryptophan residues. In addition, when excited in this region 5-hydroxytryptophan has a high limiting



anisotropy, making it useful for polarization studies. 4-Fluorotryptophan is nonfluorescent, making a "silent" analog. It also has been used in nonfluorescence experiments because of its altered ground state dipole. 7-Azatryptophan has a dramatic change in quantum yield upon exposure to water, making it potentially useful in protein folding experiments.

2.1.2 *Other Natural Fluorophores and Fluorescence Labels*

A variety of biological molecules contain naturally occurring or intrinsic fluorophores. Tryptophan is the most highly fluorescent amino acid in proteins (see sections 2.1.1 and 2.2). It is so widely used that the term "natural protein fluorescence" is almost always associated with tryptophan fluorescence. The second most fluorescent amino acid is tyrosine, but its application is mostly limited to tryptophan-free proteins. Fluorescence of phenylalanine is weak and almost never used in protein studies. Another class of natural fluorophores consists of cofactors, NADH (reduced β-nicotinamide adenine dinucleotide) being the most prominent.

The fluorescence properties of NADH have been the subject of multiple studies.[29–31] In aqueous solution the quantum yield of fluorescence is low and the average lifetime is in the subnanosecond range due to stacked conformation of the molecule. Binding to liver alcohol dehydrogenase leads to a blue-shift in absorbance and fluorescence and an increase in quantum yield (see section 3.2). This effect is enhanced by formation of a ternary complex with the substrate analog isobutyramide. The lifetime distribution for NADH shifts into a nanosecond range and undergoes a complex change in binary and ternary complex, indicating the existence of an excited state reaction.[31]

The fluorescence properties of natural chromophores are frequently inadequate for certain studies. In these cases fluorophores foreign to the system under study but displaying improved spectral properties are chosen. These extrinsic fluorophores when covalently linked to a protein (usually at a cysteine or lysine side chain) are called fluorescence labels. Modern labeling procedures in combination with mutagenesis allow labels to be introduced selectively at a specific location in a protein. The *Handbook of Fluorescent Probes and Research Chemicals*[32] provides an excellent source of information on various dyes and labeling procedures.

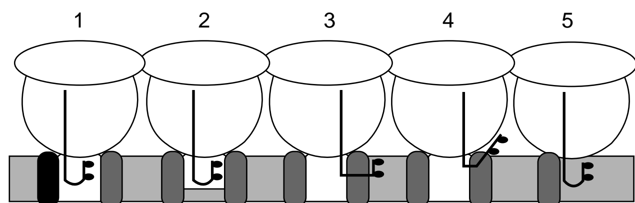


Figure 1 Example of the experimental system requiring the use of fluorescence labels in protein studies. Neither intrinsic fluorescence nor conventional labeling can specifically probe the environment of the nascent chain (solid line) in the presence of ribosome–translocon complex (large ovals). To overcome this limitation Crowley et al.(34) have developed a way to biosynthetically incorporate NBD-labeled (small ovals) lysine residues in the nascent chain. Fluorescence of NBD dye is sensitive to the polarity of its environment and to addition of water-soluble quenchers, such as iodide. Combination of steady-state and phase-modulation measurements allowed differentiation between various possibilities of organization of the translocon complex. (Reproduced by permission of Cell Press.)

In an ingenious example of the use of a fluorescence label, the fluorescence properties of 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexonic acid (NBD) were utilized to determine the environment of the nascent chain in the translocon complex(33–35) (Figure 1). Emission of NBD is very sensitive to the properties of its environment: moving from an aqueous to apolar environment increases the lifetime and the quantum yield and causes blue-shift of emission. Nascent chains, however, cannot be labeled selectively in the presence of ribosomes and endoplasmic reticulum-membrane proteins. Site-specific positioning of the probes in the nascent polypeptides can only be achieved by using aminoacyl-tRNA analogs that incorporate amino acids labeled with suitable probes into the chain as it is being synthesized. Johnson et al.(36) devised a method for preparing analogs in which probes are covalently attached to the side chain of lysine in Lys-tRNA. The validity of the approach was demonstrated by showing that the modified lysines were incorporated successfully into proteins in vitro. Since different models of translocon organization differ primarily in the exposure of the nascent chain to the cytoplasm, they could be tested directly by determining whether fluorescent dyes in the nascent chains of membrane-bound ribosomes were accessible to water-soluble quenchers in the cytoplasm. NBD dyes located at different positions along the nascent chain pathway in free ribosomes were each found to have very short fluorescence lifetimes (less than a nanosecond) and to be quenched when iodide ions were added to the solvent.(34) Thus, the nascent chain is in an aqueous environment and exposed to the cytoplasm when bound to a free ribosome. But when iodide ions were added to membrane-bound samples of the same ribosome–nascent-chain complexes, the Stern–Volmer constant for fluorescence quenching was reduced by an order of magnitude. This observation led to the conclusion that, in early stages of translocation, the nascent chain is tightly surrounded by the ribosome and membrane components of the endoplasmic reticulum.

2.2 Spectral Classes of Tryptophan Residues

The position of the maximum of the fluorescence spectrum of tryptophan residues in proteins varies from 307 to 353 nm. According to the model of discrete states of Burstein et al.(37,38) there are five most probable spectral forms of tryptophan residues (Figure 2). Formation of various exciplexes (complexes in the excited state) and subsequent dipole relaxation is believed to cause the red-shift of emission.

Spectral form A corresponds to the emission of the unperturbed indole chromophore in the extremely nonpolar environment inside the protein globule. Such emission in its pure form was found only in azurin, however the class A spectrum contributes to the total spectra of a few other proteins. Azurin, a small globular metal-binding protein, contains a single tryptophan residue that displays a rather unusual fluorescence spectrum. It is extremely blue-shifted, with a main maximum of about 307 nm, and it possesses a distinct vibrational structure. The emission from the ^1L_b state is assumed to contribute significantly in the spectrum, giving rise to mirror symmetry of emission and absorbance.

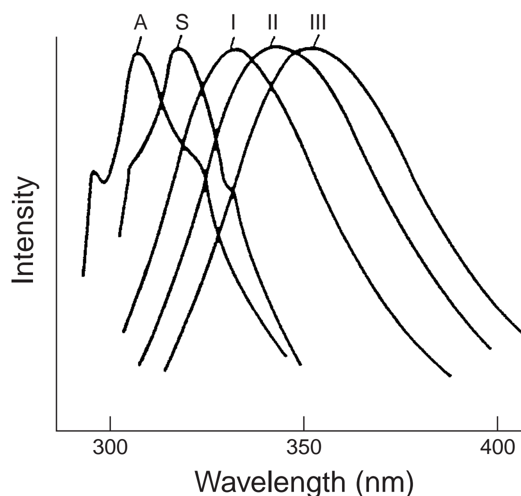


Figure 2 Normalized fluorescence spectra of tryptophan residues belonging to five spectral classes A, S, I, II and III according to Burstein.(38) Extreme variability of the emission of the indole fluorophore, depending on polarity and mobility of its environment, makes tryptophan fluorescence a sensitive tool in protein analysis. (Reproduced by permission of ONTI NCBI.)

Freezing an azurin solution does not change the position of its fluorescence spectrum.

Spectral form S corresponds to the emission of the indole chromophore located in the relatively nonpolar environment inside the protein globule and forming a 1 : 1 exciplex with some polar protein group. The S spectrum has a maximum at 316–317 nm and shoulders at 305–307 nm and 320–330 nm. It is of interest that a pure S spectrum has never been reported, but is always accompanied in proteins by a contribution from the class I spectrum (see below), which corresponds to a 2 : 1 exciplex. For example, types S and I contribute almost equally to emission of L-asparaginase.[39] It is assumed that during the excitation lifetime the 2 : 1 complex is formed to a different extent in different proteins depending on the mobility and abundance of polar groups in the tryptophan environment. Freezing of the solution does not shift class S spectra, indicating that a 1 : 1 complex can be formed at temperatures as low as −196 °C.

Spectral form I corresponds to the emission of the indole chromophore in the somewhat polar but perhaps rigid environment inside the protein globule, forming a 2 : 1 exciplex with two neighboring polar protein groups. The fluorescence spectrum of class I has a maximum at about 330 nm, width of about 50 nm and no vibrational structure. A class I spectrum is observed for actin, chymotrypsin and tetrameric melittin under high-salt conditions.

Spectral form II corresponds to the emission of the indole chromophore at the protein surface. It is assumed to be in contact with bound water and other polar groups. The fluorescence spectrum of class II is also structureless, but red-shifted as compared to class I. The position of the maximum is at about 340 nm and the width is about 55 nm. Many proteins contain tryptophan residues of this class, e.g. human serum albumin and myosin.

Spectral form III corresponds to the emission of the indole chromophore at the protein surface in contact with free water molecules. The spectrum of class III, with its maximum at about 350 nm and width of about 60 nm, nearly coincides with the spectrum of free tryptophan. Tryptophan residues of spectral class III very seldom occur in the native proteins, but are typical for unfolded states.

Due to their location on the protein interface, tryptophans of spectral classes II and III are easily accessible to external quenchers in aqueous phase. At the same time, tryptophans of classes A, S and I are located inside the protein structure and are poorly accessible to water-soluble quenchers. Freezing of the water solution of a protein shifts the fluorescence spectra of classes I, II and III towards shorter wavelengths due to the immobilization of their polar environment. It should be noted that the model of discrete states of tryptophan residues in proteins is a statistical one, i.e. tryptophan residues located in environments that result in one of the five likeliest spectral forms is more probable than finding them in intermediate environments.

Most proteins, however, exhibit spectra that contain contributions from different classes. To resolve those contributions a fitting procedure was designed based on a parameterized description of fluorescence spectra. At the heart of this analysis lies approximation of emission spectra with the log-normal distribution[40] usually expressed on the scale of wavenumbers, ν, as shown in Equations (7) and (8):

$$I(\nu) = I_{\max} \exp\left(-\frac{\ln 2}{\ln^2 \rho} \ln^2 \frac{a-\nu}{a-\nu_{\max}}\right) \quad \text{for } \nu < a \qquad (7)$$

$$I(\nu) = 0 \quad \text{for } \nu \geq a \qquad (8)$$

where $I_{\max}$ is intensity at position of maximum, $\nu_{\max}$, and $\rho = (\nu_{\max} - \nu^-)/(\nu^+ - \nu_{\max})$ is spectral asymmetry. Parameter $a = \nu_{\max} + (\nu^+ - \nu^-)\rho/(\rho^2 - 1)$, where ν^- and ν^+ are the positions on the wings of the spectrum where intensity equals half of $I_{\max}$. The total of four fitting parameters ($I_{\max}$, $\nu_{\max}$, ν^-, ν^+) per spectrum is adequate to get a unique solution, but to resolve two or more spectral components additional constraining factors are required. They are obtained from the empirical observation that width and position of the maximum of tryptophan derivatives in isotropic media are related as shown in Equations (9) and (10):

$$\nu^+ = 0.8308\nu_{\max} + 7071\,\text{cm}^{-1} \qquad (9)$$

$$\nu^- = 1.1768\nu_{\max} + 7681\,\text{cm}^{-1} \qquad (10)$$

An advanced version also utilizes spectra collected at different concentrations of quenchers to increase reliability and robustness of fit.[41]

In a simplified version of the analysis, the spectrum is fitted to a single log-normal distribution (often on a wavelength scale) to recover full width at half-height, γ, and position of maximum, $\lambda_{\max}$.[37,42] If the point on a spectral width vs position plot appears above the standard line for tryptophan in model isotropic media, several spectral classes are present. For example, a conformational change in bee venom peptide melittin can be followed by position-width analysis of steady-state spectra of Trp-19 (Figure 3). At low ionic strength melittin exists as a mainly unstructured monomer with highly exposed tryptophan (spectral class III). Additions of salt induce a monomer–tetramer transition, resulting in the removal of the tryptophan side chain from the aqueous phase (spectral class I). Intermediate states have significantly broadened spectra and follow theoretically

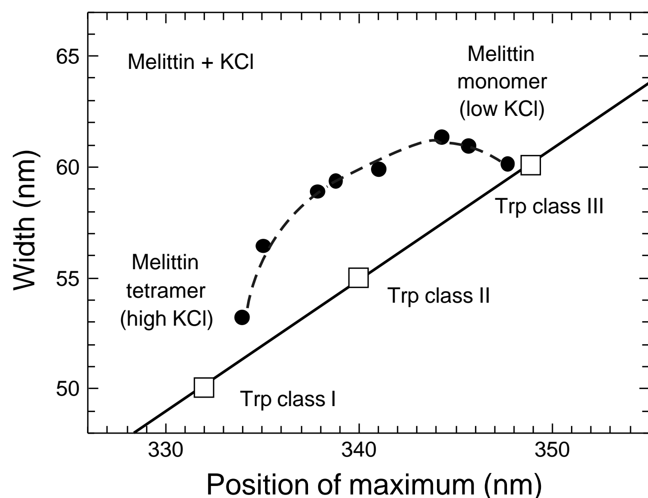


Figure 3 Example of protein conformational transition followed by spectral changes in tryptophan emission spectrum. Steady-state fluorescence spectra were fitted to a log-normal distribution to recover position of maximum and width. Open squares represent discrete states of tryptophan residues in proteins belonging to spectral classes I, II and III. Fluorescence is heterogeneous when the position–width point is above the straight line. Closed circles represent titration of melittin solution with KCl, which in this peptide causes conformational change from monomer to tetramer. This transition results in changes of exposure of the tryptophan residue from completely exposed (class III) directly to buried (class I), without going through the intermediate state (class II).

predicted semi-arc patterns.[37] Several advantages of position–width analysis over simple analysis of λ_{max} are immediately obvious. First, it is clear that spectral class II is not involved in the transition, because the spectra in the mid-transition are too broad, although they have the same maximum as II. Second, λ_{max} is not changed appreciably in the last stages of transition, and completion of conformational change is best judged by the narrowing of the spectral width. It should be emphasized, however, that despite its sensitivity the analysis of spectral position cannot be used to quantitate the conformational change or ligand binding, because λ_{max} is not a linear response function (see section 3.2). To calculate the thermodynamic parameters one should use one of the linear response spectroscopic functions as the observable, e.g. the intensity at any constant wavelength.

2.3 Protein Dynamics and Heterogeneity of Fluorescence Decay

It has long been noted that even for single tryptophan-containing proteins, fluorescence decay does not follow simple exponential kinetics.[43] Due to the nature of the lifetime analysis certain assumptions have to be made, which usually means that complex decay is assumed to be described by a number of exponential components. As instruments become more sophisticated, more components have been reported and even the distributions of lifetimes have been utilized to fit the data.[44–47] Regardless of the mode of analysis (discrete exponentials or distributions), the main empirical result is that it takes several parameters to describe fluorescence decay. Exponentials, however, do not form an orthogonal system of functions and multiple solutions can exist which fit the same data equally well. Therefore, one should use the term multi-exponential decay with caution, keeping in mind that the exact nature of nonexponentiality might not be known, and that recovered components may or may not have a physical meaning.

Interpretations of complex decay of tryptophan fluorescence fall into two main categories: ground state heterogeneity (e.g. rotamer model) and excited state reaction (e.g. relaxation model). Based mainly on model studies done with tryptophan and tyrosine derivatives, several groups have suggested that different rotameric forms are responsible for nonexponential decay.[24,48,49] In recent years there has been a breakthrough in understanding the mechanisms governing the decay of rotamers of restricted indole derivatives.[20,50,51] However, those model studies were carried out in isotropic fast-relaxing media. Proteins, in contrast, are heterogeneous systems with a hierarchy of internal motions that cover a wide range of correlation times, including the nanosecond time window of fluorescence. On the other hand, the excited state reaction approach relies on protein dynamics to convert electronically excited indole into other spectroscopic species. More specifically, the relaxation model assumes that such reaction involves reorientation of polar groups surrounding tryptophan in a protein.[52–54] Neither of the models has been unequivocally proven for any specific protein.

Recently it has been suggested that excitational dependence can be used as a criterion to distinguish between alternative mechanisms causing deviations from exponential decay in proteins.[55] This approach takes advantage of considerations developed to explain inhomogeneous broadening of the electronic spectra of dye molecules caused by the distribution in configurational energy in solvate.[56–58] Such systems are characterized by the following features: (1) decay is nonexponential, (2) decay depends on the excitation wavelength, λ_{exc}, and becomes faster at longer λ_{exc} (red-edge effect), and (3) in extreme cases a rising component in intensity (negative pre-exponential) is observed at the longer emission wavelength.

An abbreviated summary of these features for fluorescence decay in simple model systems and in proteins

Table 1 Summary of heterogeneity of fluorescence decay of indole fluorophore in model media and in proteins

System	Is fluorescence decay monoexponential?	Does decay depend on excitation wavelength?	Is the rising intensity component observed?	Nature of heterogeneity of decay
Indole in water or methanol	Yes	No	No	None (no rotamers, fast relaxation)
Indole in 40% glycerol	No (2 components)	Yes	No	Mild relaxation effects
Indole in 90% glycerol	No (3 components)	Yes	Yes	Relaxation effects
Trp in water	No (2 components)	No	No	Rotamers
Trp in 40% glycerol	No (3 components)	Yes	No	Rotamers + mild relaxation effects
Proteins (single tryptophan residue)	No (2–4 components, usually 3)	Both possibilities can be observed	No (with rare exceptions)	? (possible combination of several mechanisms)

is presented in Table 1 (Ladokhin, unpublished).[55] Slowing of dipolar relaxation by additions of glycerol leads to progressive heterogeneity and excitational dependence in fluorescence decay of the indole molecule, which obviously lacks rotameric forms. Note that deviation from exponentiality starts at low glycerol concentrations, before the negative pre-exponential could be observed under commonly used instrumental conditions (the width of the excitation pulse was 60 ps). As described above (see section 2.1.1), tryptophan zwitterion in aqueous solution shows a double exponential decay due to rotameric forms.[24] In this case, however, the decay is independent of the excitation wavelength. When a tryptophan molecule is placed under conditions of mild viscosity, decay becomes more complex, and in this case excitational dependence can be detected. In proteins the excitation dependence can be small or significant and the negative exponential normally is not observed unless four exponential components are used in the analysis, and then only for a few proteins. Melittin in methanol, when it is helical and monomeric, constitutes an exception when a negative pre-exponential is observed with one of the three components.[55] This overall behavior of proteins is consistent with contributions from both ground state heterogeneity and excited state reactions.

The model studies presented above indicate the importance of protein dynamics for interpretation of fluorescence decay in proteins. This is also true for other fluorescence parameters, such as dynamic depolarization, biomolecular quenching rate, temperature-induced quenching in the native state, red-edge excitation shift, distribution of distances calculated in energy transfer experiments, etc. In a sense all protein fluorescence spectroscopy is influenced by the dynamic nature of protein. Further understanding of this link will allow one to solve the reverse task and to determine dynamic parameters of protein structure from its fluorescence.

3 FLUORESCENCE STUDIES OF PROTEIN CONFORMATION AND INTERACTIONS

3.1 Protein Folding

Fluorescence spectroscopy plays a major role in protein folding studies.[59,60] For example, the fluorescence increase of 8-anilino-1-naphthalensulfonic acid was used to identify the molten globule state during refolding of various proteins,[61] and intrinsic fluorescence, along with other methods, was utilized to provide evidence for a two-state transition in chymotrypsin inhibitor.[62] A variety of fluorescence observables can be used to follow denaturation or renaturation of the protein native structure induced by changes in temperature, pH or additions of solutes. One such example has already been discussed in previous sections – changes in the emission bandshape following tetramerization of melittin was presented in Figure 3 (see section 2.2). Another example is the change in emission maximum and polarization of tryptophan fluorescence of apohorseradish peroxidase upon addition of a denaturing agent, guanidinium chloride.[63] Unfolding of this protein results in increased exposure of a single tryptophan residue to aqueous environment revealed by the fluorescence red-shift (Figure 4). Increased mobility of the tryptophan due to the loss of native packing contacts upon denaturation results in the parallel loss of emission polarization. Note that an excitation wavelength of 300 nm was used for polarization measurements to avoid strong depolarization (insert, Figure 4), due to overlapping ^1L_a and ^1L_b transitions at the shorter wavelengths (see section 2.1.1). In addition to emission maximum and polarization, a number of other parameters (e.g. intensity, lifetime distribution, Stern–Volmer constant, efficiency of the radiationless energy transfer) can be utilized in steady-state protein folding studies. But the advantages of fluorescence studies of folding are demonstrated most vividly, perhaps, with kinetic measurements.

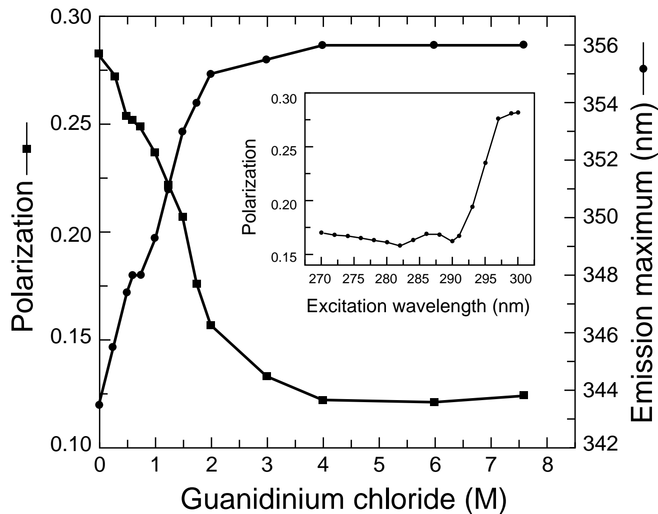


Figure 4 Example of chemical-induced protein unfolding studied by fluorescence spectroscopy.[63] Additions of guanidinium result in red-shift of fluorescence and in decrease of emission polarization of a single tryptophan in the apohorseradish peroxidase. Insert shows the excitation polarization spectrum. (Reproduced by permission of the Biophysical Society.)

Among the spectroscopic methods used in combination with stopped-flow mixing, fluorescence is probably the simplest, yet most sensitive and widely employed. Recent years have been marked by considerable advances in both mixing techniques and kinetic acquisition of spectroscopic data. Application of advanced continuous-flow mixing to follow the energy transfer from tryptophan and tyrosines to heme in cytochrome *c* has allowed probing of the barrier-free submillisecond transition between the initially collapsed state and the native state. Using a combination of both continuous-flow microsecond mixing and conventional stopped-flow mixing, Shastry et al.[64] demonstrated how continuous kinetic data from 50 μs to >10 s after initiation of the folding event can be obtained (Figure 5). Ramsay et al.[65,66] have developed a fluorimeter/CD photometer hybrid allowing global changes in secondary structure and local changes in the tryptophan environment to be determined simultaneously for the same sample.

Beechem et al.[67–69] have pioneered the development of another important technical advance in spectroscopic analysis of fast kinetic effects by combining the advantages of time-resolved spectroscopy with kinetic stopped-flow measurements. This double-kinetics technique achieves simultaneous measurement of changes in fluorescence on both the picosecond/nanosecond and millisecond timescale. After initiation of folding the decay of tryptophan anisotropy is measured in millisecond intervals of time, thus allowing the determination

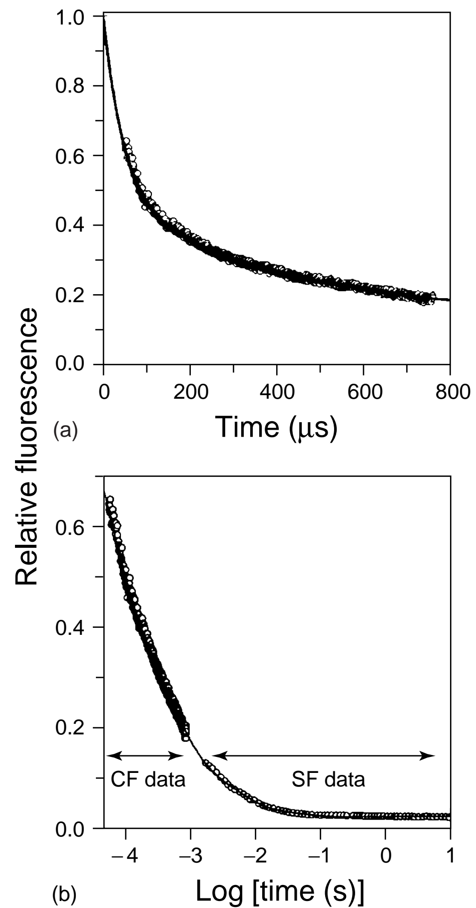


Figure 5 Example of protein folding kinetic measurement utilizing tryptophan fluorescence.[64] (a) Refolding of acid-unfolded horse cytochrome *c* was followed on the submillisecond timescale in a continuous flow capillary mixing apparatus. (b) Combination of continuous flow (CF) and conventional stopped-flow (SF) experiments allows collection of data over a wide range of time (symbols) and determination of the five kinetic phases with correlation times ranging from tens of microseconds to tens of milliseconds (solid line). (Reproduced by permission of the Biophysical Society.)

of tryptophan rotation mobility "in real time" during protein refolding. This allows ascertaining the timescale associated with tryptophan "packing" into intermediate structures and eventually into the final native state. Such experiments can also follow the changes in lifetime due to different fluorescence quenching in the folded and unfolded state. If two fluorophores capable of making a donor–acceptor pair are introduced, such studies can be used to determine the changes in distance between labeled sites on the millisecond timescale.

In addition to the water-soluble systems discussed above, fluorescence spectroscopy is now being employed to study folding of membrane proteins[70–74] (see section 3.3).

3.2 Protein–Ligand Interactions

There are two principal possibilities that can be exploited to study protein–ligand interactions. The fluorescence of either the protein or the ligand can change upon binding. These two situations require somewhat different data analysis procedures.[75] When changes in the fluorescence of a protein are observed, the fluorophore may be one or more of the intrinsic tryptophan residues, or it may be the covalently attached or tightly bound fluorophore.

Tryptophan is a relatively sensitive fluorophore. Its spectrum can vary more than 40 nm (see section 2.2) and its quantum yield in proteins and peptides ranges from virtually zero to almost 0.5. The latter change is usually accompanied by a corresponding variation in the excited state lifetime. The binding of a ligand to a protein may directly affect the fluorescence of a tryptophan residue by acting as a quencher (i.e. by a collisional or energy transfer mechanism) or by physically interacting with the fluorophore and thereby changing the polarity of its environment and/or its accessibility to solvent. Alternatively, a ligand may bind at a site remote from the tryptophan residue and act via a generalized mechanism by inducing protein conformational change, which alters the microenvironment of the tryptophan. Both direct and generalized effects may result either in enhancement or quenching of fluorescence and/or in shifts in the spectrum to the red or blue. If there are multiple tryptophan residues in a protein, this will make it difficult to assign any fluorescence changes to a specific residue. To overcome this limitation, structural studies are now being conducted on single tryptophan-containing mutants. However, for many other purposes, e.g. the thermodynamics of ligand binding, the presence of multiple fluorophores is a minor consideration. More important is the fact that the observable be a linear-response function.

3.2.1 Linear-response Spectroscopic Techniques

Consider the simple case of a two-state equilibrium (the general case is described by Toptygin and Brand[76]) that can be followed by a change in a certain spectroscopic parameter S that changes from a value S_{min} in the absence of binding to S_{max} when all of the molecules are bound. The fractional change in the spectroscopic parameter f_{signal}, defined as $f_{signal} = (S - S_{min})/(S_{max} - S_{min})$, is often used to characterize the extent of completion of the binding reaction. The fraction of bound molecules $f_{bound} = P_{bound}/P_{total}$ will coincide with f_{signal} only if S is a linear-response function. For that case, the signal observed can be represented as a linear combination of molar fractions of bound and free molecules weighted by their corresponding molar spectroscopic characteristics, s_{bound} and s_{free}, as shown in Equation (11):

$$\frac{S}{P_{total}} = f_{bound}s_{bound} + f_{free}s_{free} = f_{bound}s_{bound} + (1 - f_{bound})s_{free} \quad (11)$$

Not all spectroscopic data satisfy this equation. For example, the following spectroscopic parameters are not linear-response functions and therefore cannot be utilized for measurements of binding: position of maximum of the fluorescence spectrum and related parameters such as center of weight, center of the chord at any intensity level, or ratio of intensities on the wings; fluorescence polarization (or anisotropy); intensity at the maximum of the spectrum; the phase ϕ and modulation m from frequency domain fluorimetry; fluorescence decay curve collected to a constant peak; and transmittance from spectrophotometric measurements. Parameters that are linear-response functions and that can be utilized for measurements of binding include the following: fluorescence quantum yield; steady-state intensity at a constant wavelength I_{ss}; $I_{ss}m\cos\phi$ and $I_{ss}m\sin\phi$;[76] fluorescence decay curve collected to a constant time; vertical and horizontal components of fluorescence intensity (and of any other angle too); and absorbance from spectrophotometric measurements.

In addition to general rules that apply to all binding experiments, there are some peculiarities relevant to specific aspects of experimental schemes. For example, in membrane partitioning experiments there is an explicit assumption that s_{bound} does not depend on the completion of binding or on the number of bound molecules per lipid vesicle. However, in the case of fluorescence, the intensity can be a nonlinear function of bound molecules due to nonradiative energy homotransfer or other self-quenching mechanisms. This possibility is often overlooked during the analysis of complex membrane partitioning. Various spectroscopic and nonspectroscopic methods to quantitate binding to membranes are discussed elsewhere.[77]

Toptygin and Brand[76] have developed special software to fit multiple families of titration data obtained with any of the linear-response spectroscopic techniques. The SPECTRABIND program uses model equilibrium equations as constraints to determine the basic components associated with the actual chemical species and their concentrations. A statistical test is used to discriminate between adequate and inadequate models, the decision being independent of any knowledge and/or assumptions about the spectroscopic characteristics of chemical species. This approach is aimed at systems involving multiple interactions, where the changes in spectroscopic signal cannot be attributed to a single interaction. An example

of the application of SPECTRABIND to the interaction of horse liver alcohol dehydrogenase with NADH and isobutyramide is given in Figure 6.[76] Fluorescence spectra of NADH in various binary and ternary complexes recovered from the same titration data sets using two different binding models are presented. Both models assume that the two binding sites (one on each of the identical subunits of the protein dimer) are identical and independent in terms of association constants. Figure 6(a) contains results obtained under the additional generic assumption that binding to the two sites is also spectroscopically independent. The three essential spectroscopic species correspond to a free NADH (N), its binary complex with the protein subunit (SN), and its ternary complex with additional isobutyramide (SNI). Protein (S) and isobutyramide (I) by themselves produce negligible signal, while the background signal (B) contains a sharp peak of Raman scattering band of water. The model from Figure 6(b) does not assume spectroscopic independence of binding sites and contains multiple species of various combinations of the entire dimeric protein (P) with one or two NADH and isobutyramide molecules. The association constants for each binding step are also determined.[76] This example illustrates the ability of the SPECTRABIND program to analyze complex binding equilibria.

The basic limitation of spectroscopic techniques for studying protein–ligand interactions is the necessity for a sufficiently strong change in some spectroscopic signal. The lack of an adequate signal can hinder rigorous quantitative determination of binding. To overcome this limitation in studies of nucleic acid-binding proteins, Jezewska et al.[78,79] applied an inventive approach that takes advantage of the strong fluorescence of the reference ligand. An example of application of the Macromolecule Competition Titration method for binding of nonfluorescent single-stranded nucleic acid poly(dA) to DnaB helicase is presented in Figure 7. The observed fluorescence signal was due to the poly(dεA), an etheno derivative of poly(dA), used as a reference. This method allows the determination of absolute average binding density and construction of a model-independent true binding isotherm.

3.3 Membrane Proteins and Peptides

Determination of membrane organization and dynamics is one of the most challenging problems of structural biology because many methods (even of low resolution) developed for water-soluble systems are not directly applicable to membranes. For example, while the position of the fluorescence spectrum of tryptophan in a globular protein indicates a degree of exposure to the aqueous phase, it will not be sensitive to exposure to the

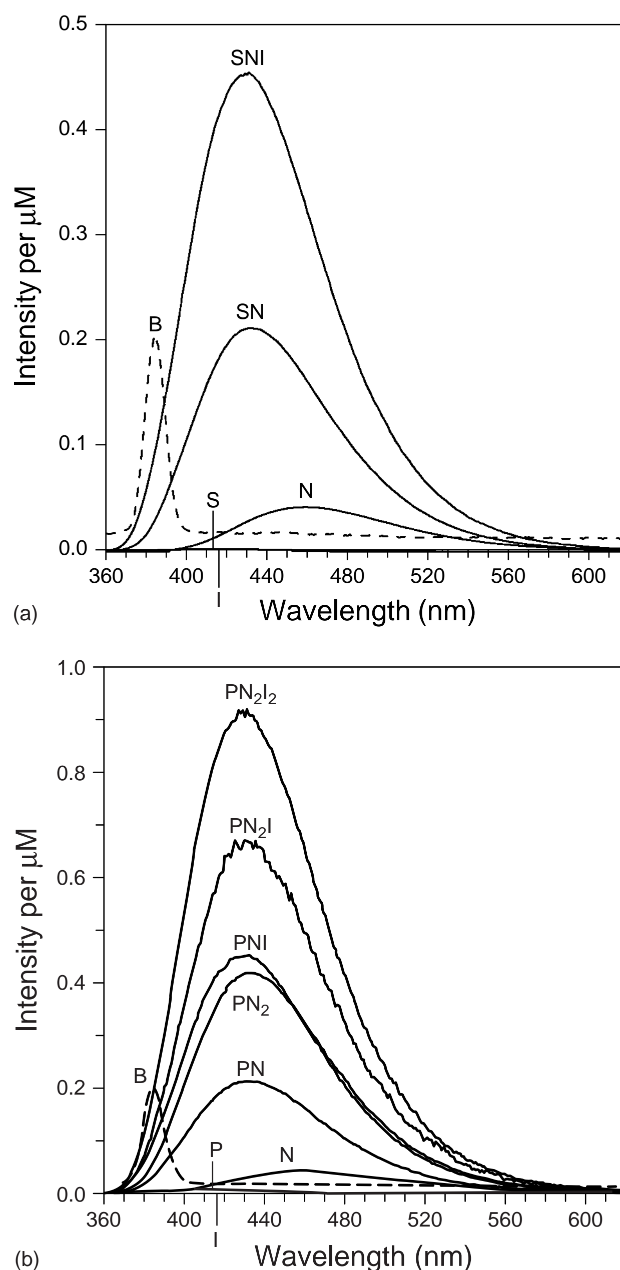


Figure 6 Example of the protein–ligand binding study followed by fluorescence. Individual spectra of NADH when free in solution (N), bound in a binary complex with horse liver alcohol dehydrogenase ((a) SN; (b) PN and PN_2), and in a ternary complex in the presence of isobutyramide ((a) SNI; (b) PNI, PN_2I and PN_2I_2), along with corresponding equilibrium binding constants, were obtained with the help of the SPECTRABIND program of Toptygin and Brand.[76] Fluorescence spectra presented in (a) and (b) are obtained from the same titration data sets using two different binding models. Both models assume that the two binding sites (one on each of the identical subunits of the protein dimer) are identical and independent in terms of association constants. Model (a) also assumes that binding to the two sites is also spectroscopically independent. See text and Toptygin and Brand[76] for details. (Reproduced by permission of Academic Press, Inc.)

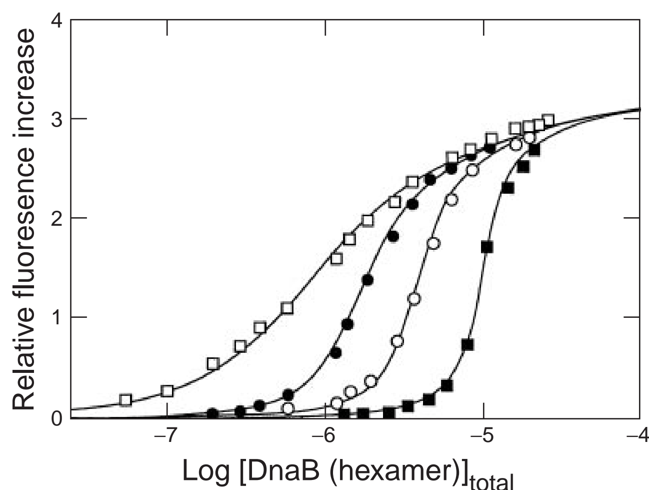


Figure 7 Example of protein–ligand interactions followed by means of fluorescence spectroscopy. The exceptional sensitivity of fluorescence is utilized in a variety of binding studies using one of the linear-response spectroscopic functions (e.g. intensity at a constant wavelength). Normally the fluorescence of the protein or ligand is altered as a result of the interaction. Jezewska and Bujalowski[78] have introduced a Macromolecule Competition Titration Method to study those interactions that do not directly result in such changes of fluorescence. Instead, the fluorescence of the reference ligand (an etheno derivative of a polynucleotide in this case) is measured in the presence of various concentrations of the ligand under study (nonlabeled polynucleotide). Fluorescence increase, plotted as a function of the protein concentration (DnaB helicase, symbols), is fitted using a binding model (solid lines) to calculate various parameters. Binding constants, parameters of cooperativity of binding and stoichiometry can be studied by fluorescence titration methods. (Reproduced by permission of the American Chemical Society.)

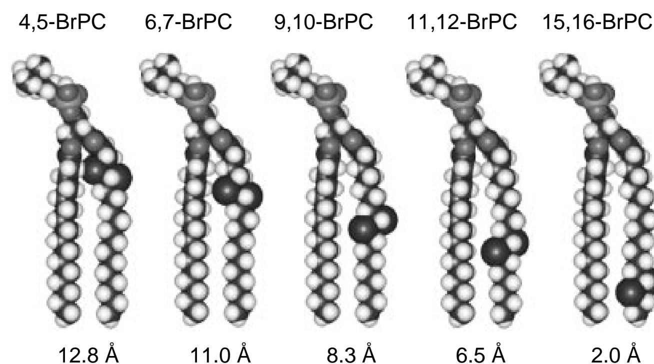


Figure 8 Set of bromolipids utilized to examine membrane penetration of proteins and peptides. Selective labeling of different positions along one of the acyl chains with bromine atoms, which are capable of quenching fluorescence via dynamic collisional mechanism,[42] allows the transverse position of the fluorophore to be determined. Two bromine atoms (large spheres) per lipid are attached in neighboring positions. This "molecular ruler" has been calibrated in an independent diffraction experiment yielding the distances (given in angstroms) from bromines to the bilayer center.[86] Depth-dependent fluorescence quenching profiles measured utilizing bromolipids can be evaluated with the help of distribution analysis[70,87,88] (see Figure 9 for examples) to obtain a variety of structural information.

lipid phase in a membrane-bound protein. Therefore, new approaches should be developed for structural studies in membranes. The depth-dependent fluorescence quenching technique is a useful tool to explore the structure of membrane proteins and peptides along the depth coordinate.[74,80–85] To achieve this objective it utilizes lipids with bromine atoms (Figure 8) or spin labels selectively attached to certain positions along acyl chains. This is a dynamic quenching resulting in decrease of both intensity and lifetime.[42,71]

In a depth-dependent fluorescence quenching experiment one normally determines the fluorescence intensity, F, of a probe as a function of the known depth of the quencher, h. Data are usually normalized to the intensity in the absence of quenching, F_0. Distribution analysis is one of the methods that can be used to quantitate the quenching in order to extract information on membrane penetration. It assumes that the quenching profile can be approximated by a symmetrical double-Gaussian function, which accounts for *cis*- and *trans*-leaflet quenching,[70,87] as shown in Equations (12) and (13):

$$\ln\frac{F_0}{F(h)} = G(h,\sigma,S) + G(-h,\sigma,S) \qquad (12)$$

$$G(h,\sigma,S) = \frac{S}{\sigma\sqrt{2\pi}}\exp\left[-\frac{(h-h_\mathrm{m})^2}{2\sigma^2}\right] \qquad (13)$$

where h_m, σ and S are the three fitting parameters: mean position, dispersion and area, respectively.

The position of the maximum represents the most probable transverse location of the fluorophore, while dispersion arises from the several broadening terms, such as finite size of the probe and the quencher and fluctuational distribution of their depth due to thermal motion. Multiple conformations will result in additional broadening of the profile. Equation (14) shows that the area under the quenching profile is a product of the inherent quenching constant, γ, determined by the nature of the quenching mechanism, excited state lifetime in the absence of quenching, τ, the degree to which the probe is exposed to a lipid phase, w, and the concentration of the quencher, C:

$$S = \gamma w \tau C \qquad (14)$$

Variation of exposure arising from the shielding of tryptophan side chains by the protein moiety provides important structural information on protein conformation

in the lipid bilayer. Equation (15) shows that the relative exposure, Ω, can be estimated as the ratio of absolute exposures of tryptophan residue in a protein, w_P, to that in a model compound (e.g. tryptophan octyl ester[42,88]), w_M:

$$\Omega = \frac{w_P}{w_\mathrm{M}} = \frac{S_P \tau_\mathrm{M}}{S_\mathrm{M} \tau_P} \approx \frac{S_P Q_\mathrm{M}}{S_\mathrm{M} Q_P} \quad (15)$$

If the lifetime measurements are not available, the ratio of τ values can be approximated by the ratio of quantum yields, Q, of a protein and a model compound in a nonquenching lipid membrane.

Application of the distribution analysis technique to a mutant cytochrome b_5 and to a model membrane-spanning peptide are presented in Figure 9. A single fluorophore of the membrane-binding peptide of cytochrome b_5 mutant, Trp-108, was found to be located close to the membrane interphase and its distribution along the depth coordinate is relatively narrow, suggesting the lack of conformational freedom.[70,87] The relative exposure of Trp-108 to lipid phase equals 0.66, as compared to tryptophan octyl ester. A single tryptophan in a membrane-spanning peptide is located deep in the hydrophobic core of the bilayer. For this peptide, the use of a double-Gaussian fitting function was especially important due to strong trans-leaflet quenching. Thus for this peptide the unimodal distribution of transverse position (Figure 9) differs from that of a quenching profile.

It has been demonstrated that all three parameters – average depth of the tryptophan, the width of its transverse distribution and the degree of its exposure to the lipid phase – are different in various proteins and peptides. Studies of melittin and various mutants of cytochrome b_5 and of outer membrane protein A indicate that these parameters can be altered by temperature-induced conformational change or during the kinetic insertion and folding.[70–73,88]

Another important aspect of the structural characterization of membrane proteins and peptides is determination of their topology. The critical issue is: Does a particular peptide equilibrate freely across the bilayer, form a stable transmembrane structure, or remain only on one surface? Recently Wimley and White[91] have developed a method that uses fluorescence quenching arising from resonance energy transfer for determining the topology of the tryptophan residues of peptides partitioned into phospholipid bilayer vesicles. This is accomplished through the use of a novel lyso-phospholipid quencher, lyso-methylcoumarin (LysoMC). Methylcoumarin quenches the fluorescence of membrane-bound tryptophan by the long-range resonance energy transfer mechanism with an apparent Förster distance that is comparable to the thickness of

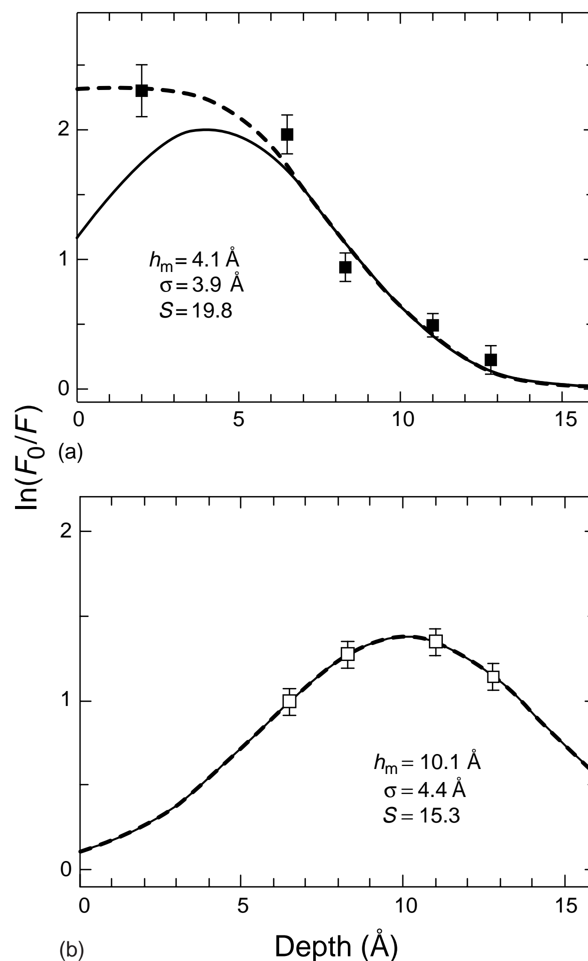


Figure 9 Examples of structural studies of membrane proteins and peptides using depth-dependent fluorescence quenching. Symbols represent quenching data for (a) membrane-spanning peptide[89] and (b) cytochrome b_5 mutant[90] obtained with a set of bromolipids (Figure 8). Dashed lines are the fitting curves of the distribution analysis[70,87,88] utilizing Equations (12) and (13). Solid lines are the individual Gaussian components with three parameters h_m, σ and S, representing average position of the fluorophore in the lipid bilayer, heterogeneity of its transverse penetration, and quenching efficiency, respectively.

the hydrocarbon core of a lipid bilayer (~25 Å). Consequently, the methylcoumarin acceptor predominantly quenches tryptophans that reside in the same monolayer as the probe. The topology of a peptide's tryptophan in membranes can be determined by comparing the quenching in symmetric and asymmetric LysoMC-labeled vesicles (Figure 10).

Both structural and thermodynamic studies on membrane proteins are clearly lagging behind those of their soluble counterparts. Only recently have the first basic principles of integral protein structure and stability begun to emerge.[77,92] The general reason for this is that the lipid bilayer membrane appears to be too large and

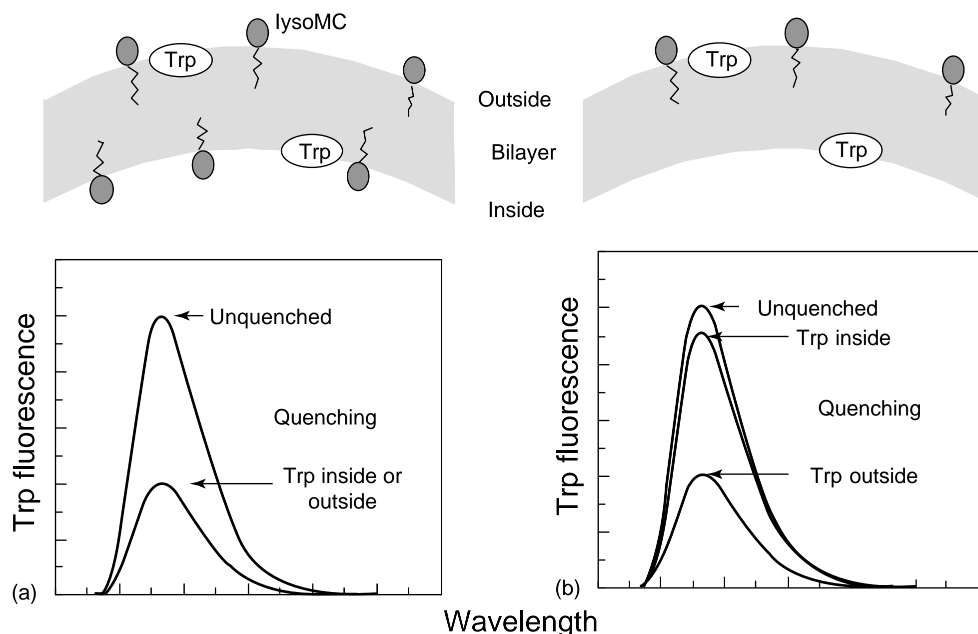


Figure 10 Schematic representation of the resonance energy transfer method of Wimley and White[91] for the determination of the topology of tryptophan in a membrane protein. LysoMC, which acts as the acceptor of electronic energy of tryptophan via long-range Förster mechanism, can be easily incorporated into large unilamellar vesicles either symmetrically (a) or asymmetrically (b). Comparison of the efficiency of energy transfer reveals the topology of the tryptophan.

slow-tumbling for solution NMR studies and too "two-dimensional" for X-ray crystallography. These limitations hindering applicability of high-resolution structural techniques do not compromise in principle the application of fluorescence and other spectroscopic methods (e.g. EPR[93]). Therefore, in addition to its traditional role as a technique sensing dynamic fluctuational aspects of protein structure and its kinetic metamorphoses, fluorescence spectroscopy is assuming a new role as a direct structural tool in studies of membrane proteins.

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ABBREVIATIONS AND ACRONYMS

CD	Circular Dichroism
EPR	Electron Paramagnetic Resonance
IR	Infrared
LysoMC	Lyso-methylcoumarin
NADH	Reduced β-Nicotinamide Adenine Dinucleotide
NBD	6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexonic Acid
NMR	Nuclear Magnetic Resonance

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