Fluorescence of Membrane-bound Tryptophan Octyl Ester: a Model for Studying Intrinsic Fluorescence of Protein–Membrane Interactions

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Fluorescence of Membrane-Bound Tryptophan Octyl Ester: A Model for Studying Intrinsic Fluorescence of Protein-Membrane Interactions

Alexey S. Ladokhin* and Peter W. Holloway‡
*Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kiev 252030, Ukraine, and ‡Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908 USA

ABSTRACT The fluorescence of a membrane-bound tryptophan derivative (tryptophan octyl ester, TOE) has been examined as a model for tryptophan fluorescence from proteins in membrane environments. The depth-dependent fluorescence quenching of TOE by brominated lipids was found to proceed via a dynamic mechanism with vertical fluctuations playing a central role in the process. The activation energy for the quenching was estimated to be 1.3 kcal/mole. The data were analyzed using the distribution analysis (DA) method, which extends the conventional parallax method to account more realistically for the transbilayer distributions of both probe and quencher and for possible variations in the probe's accessibility. DA provides a better fit than the parallax method to data collected with TOE in membranes formed of lipids brominated at either the 4,5, the 6,7, the 9,10, or the 11,12 positions of the *sn*-2 acyl chain. DA yields information on the fluorophore's most probable depth in the membrane, its conformational heterogeneity, and its accessibility to the lipid phase. Previously reported data on cytochrome b_5 and melittin were reanalyzed together with data obtained with TOE. This new analysis demonstrates conformational heterogeneity in melittin and provides estimates of the freedom of motion and exposure to the lipid phase of membrane-embedded tryptophans of cytochrome b_5.

INTRODUCTION

Determination of the structure of membrane proteins is one of the most challenging problems of structural biology because many of the high resolution methods developed for water-soluble systems are not directly applicable to membranes. Consequently, there has been increasing use of fluorescence spectroscopy in studies of membrane-bound proteins and peptides (London and Feigenson, 1981a,b,c; Chatelier et al., 1984; Markello et al., 1985; Kleinfeld, 1985; Vogel and Jahnig, 1986; Talbot et al., 1987; Berkhout et al., 1987; Ladokhin et al., 1988, 1991, 1992a,b,c, 1993a,b; Bolen and Holloway, 1990; De Kroon et al., 1990; Clague et al., 1991; Chattopadhyay and McNamee, 1991; Chung et al., 1992; Gonzalez-Manas et al., 1992, 1993; Valenzuela et al., 1994; Jones and Gierasch, 1994; Rodionova et al., 1995). Despite the popularity of the method, it appears that very little attention has been paid to the influence of the lipid bilayer itself on the spectroscopic properties of natural fluorophores. The successful interpretation of fluorescence (mainly that from tryptophan residues) of water-soluble proteins is based on model studies performed with tryptophan derivatives in various isotropic media. Extension of such a strategy to membrane-bound proteins often is based on an oversimplified presentation of a lipid bilayer as a hydrophobic phase. Recent studies of Wimley and White (1993) suggest that specific bilayer effects, distinct from the hydrophobic effect, should be considered in a description of membrane interactions.

In the present study we concentrate our efforts on the characterization of specific spectroscopic features of the fluorescence of the indole chromophore inserted into a bilayer. The need for understanding these features can be illustrated with the following example. Consider a water-soluble protein undergoing a conformational change that results in the alteration of the tryptophan's position within the protein interior. This change in exposure could be monitored either by change in the spectral position of the emission band (Burstein, 1973; Burstein et al., 1973) or by quenching with water-soluble quenchers (Eftink and Ghiron, 1981). Both of these approaches should be revised for membrane-bound proteins as they would not distinguish between tryptophan being inside the protein or inside the lipid phase.

We suggest that the depth-dependent fluorescence quenching caused by the quenchers attached to specific positions along the lipid acyl chains can yield indispensable structural information on membrane-bound proteins and peptides. We discuss in detail a comprehensive procedure for quantitating the depth-dependent fluorescence quenching in membranes, which is based on physically relevant assumptions and provides parameters with a clear physical meaning. The distribution analysis (DA) technique (Ladokhin, 1993; Ladokhin et al., 1993a) employed here provides not only the most probable depth of the fluoro-

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Address reprint requests to Dr. Alexey Ladokhin, Department of Physiology and Biophysics, University of California, Irvine, College of Medicine, Irvine, CA 92717. Tel.: 714-824-6993; Fax: 714-824-8540; E-mail: ladokhin@helium.biomol.uci.edu.

Abbreviations used: TOE, DL-tryptophan octyl ester; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; 4,5-, 6,7-, 9,10-, or 11,12BRPC, 1-palmitoyl-2(dibromostearoyl)phosphatidylcholine with bromine atoms at the 4,5, the 6,7, the 9,10, or the 11,12 positions, respectively; DBRPC, 1,2-di(dibromostearoyl) phosphatidylcholine with bromine atoms at the 9,10 positions; DMPC, 1,2-dimyristoylphosphatidylcholine; DA, distribution analysis; PM, parallax method.

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phore but also allows the estimation of its conformational heterogeneity and accessibility to the lipid phase.

However, to be able to interpret correctly fluorescence data collected on proteins in membranes, one has to study a model compound in a lipid environment first. We chose a tryptophan derivative with a hydrocarbon chain attached, TOE, for this purpose.

The location of TOE in the membrane was determined by depth-dependent fluorescence quenching utilizing membranes formed of lipids labeled with bromine atoms at either the 4,5, the 6,7, the 9,10, or the 11,12 positions of the *sn*-2 acyl chain. It was found that the DA provides a more accurate fit, as compared with the parallax method (PM) (Chattopadhyay and London, 1987; Abrams and London, 1992, 1993), to the complete set of data available. This results in more accurate determination of the values for the depth of penetration of the fluorophore into the membrane. The mean depth for the TOE distribution was found to be 11 Å from the bilayer center and to change little with temperature. The large width of the TOE depth distribution reported here makes its fluorescence sensitive to both the acyl chain phase transition and to dipolar relaxation of the head groups. It was shown that quenching occurs via a dynamic mechanism, with vertical fluctuations playing a central role in the process. From the temperature dependence of the quenching efficiency the activation energy for the quenching was estimated to be 1.3 kcal/mole.

Based on the results of the TOE study, new information was extracted from the fluorescence data on several proteins that had previously been published (Ladokhin et al., 1991, 1992c, Ladokhin and Holloway, 1995). This new analysis demonstrates conformational heterogeneity of a protein molecule (melittin) and provides estimates of the freedom of motion and exposure to the lipid phase of membrane-embedded tryptophans of cytochrome b_5.

Finally, we report here a number of fluorescence properties of membrane-bound TOE, such as lifetime, quantum yield, anisotropy, spectral distribution, and "red edge" shift, that could be used as reference points for a variety of studies involving fluorescence of tryptophan residues in membrane proteins.

A preliminary account of this work has appeared elsewhere (Ladokhin et al., 1992a, 1993a).

MATERIALS AND METHODS

POPC was from Avanti Polar Lipids, Inc. (Pelham, AL) and BRPCs were synthesized as described previously (Markello et al., 1985). Lipid vesicles were prepared by sonication as described previously (Markello et al., 1985). The size of the vesicles was determined by 90° quasi-elastic laser light scattering (Nicomp Instruments, Inc., Santa Barbara, CA). The diameter distribution function was centered at 95–115 nm with a standard deviation of 30–40 nm for all of the samples. TOE was from Sigma Chemical Co. (St. Louis, MO). Samples were prepared by mixing the TOE solution in 10 mM HEPES buffer containing 0.1 mM EDTA, pH 7.1, with enough of a stock solution of sonicated vesicles to achieve the desired lipid/probe molar ratio (400 for time-resolved experiments or 1000 for other measurements). The final concentration of TOE was 2–5 μM.

Steady-state fluorescence measurements were made with an SLM 8000c spectrofluorometer (Urbana, IL). An excitation of 280 nm was used unless otherwise indicated. Both excitation and emission slits were not wider than 4 nm. Normally from 5 to 15 spectra were averaged to get an adequate signal-to-noise ratio. To improve accuracy in determining the spectral position, the following procedure was utilized: emission spectra were recorded from 290 to 500 nm with a 1-nm increment and, after background correction, were fitted to a log-normal distribution (Ladokhin et al., 1991):

$$I(\lambda) = I_0 \exp - \{\ln 2(\ln \rho)^{-2}[\ln(1 + (\lambda - \lambda_{max}) \times (\rho^2 - 1)/(\rho\Gamma))]^2\}$$
$$\text{for } \lambda > \lambda_{max} - [(\rho\Gamma)/(\rho^2 - 1)] \quad (1)$$
$$I(\lambda) = 0 \qquad \text{for } \lambda < \lambda_{max} - [(\rho\Gamma)/(\rho^2 - 1)],$$

where λ_{max} is the wavelength of maximal intensity of the spectrum, I_0 is the maximal intensity observed at λ_{max}, Γ is the width of the spectrum at half of I_0, and ρ is the parameter of asymmetry. The center of weight of the log-normal distribution was determined by numerical integration.

To eliminate scattering artifacts caused by the lipid vesicles, the samples were subjected to the dilution technique of Eisinger and Flores (1985). The difference between the corrected and uncorrected intensities was 10–20%. For all types of fluorescence measurements, a magic angle configuration of polarizers was used to exclude the artifacts associated with dynamic depolarization.

The depth-dependent quenching data obtained with different BRPCs were fitted to the following:

$$F_0/F(h) = \exp\{G(S, \sigma, h - h_m)\}, \quad (2)$$

where $F(h)$ is a set of intensities measured as a function of vertical distance from the bilayer center to the quencher (h). Three parameters of the Gaussian distribution (G), most probable position of fluorophore (h_m), dispersion (σ), and area (S), were recovered from measurements with four different brominated lipids, with known position of quenching atoms (h) (McIntosh and Holloway, 1987) and a control measurement with POPC (F_0). The justification for Eq. 2 is given in the Appendix.

Time-resolved fluorescence decays were collected with a spectral resolution of 16 nm using a time-correlated single photon counting apparatus as described earlier (Badea and Brand, 1979). The cavity-dumped output of a synchronously pumped rhodamine-6G dye laser was used to generate a laser pulse, which was then frequency-doubled to a wavelength of 285 nm. Experimental data were analyzed by the nonlinear least-squares method assuming that fluorescence decay can be represented as a sum of exponential components: $I(t) = \Sigma\alpha_i \exp(-t/\tau_i)$. The goodness of fit was assessed by χ^2 criteria (χ^2 ranged from 1.03 to 1.25) and randomness of residuals and their autocorrelation. To recover decay-associated spectra, a series of decays measured at different wavelengths was analyzed globally by linking the lifetime and allowing α values to float independently. The pre-exponential factors were normalized to yield intensity, calculated as $\Sigma\alpha_i\tau_i$, which is proportional to the steady-state intensity measured independently. For the presentation in the table the pre-exponential terms were normalized to a sum of 100%.

RESULTS

The fluorescence of TOE is sensitive to membrane binding (Fig. 1). Upon incorporation into POPC vesicles the fluorescence intensity of the TOE increases almost three times and the spectrum is blue-shifted. The quantum yield of POPC-bound TOE at 25°C was found to be 95 ± 5% of that of the aqueous tryptophan. From the data presented in Fig. 1 it is obvious that the binding is tight and the sample with a lipid/probe ratio of 100 contains only a small amount of a

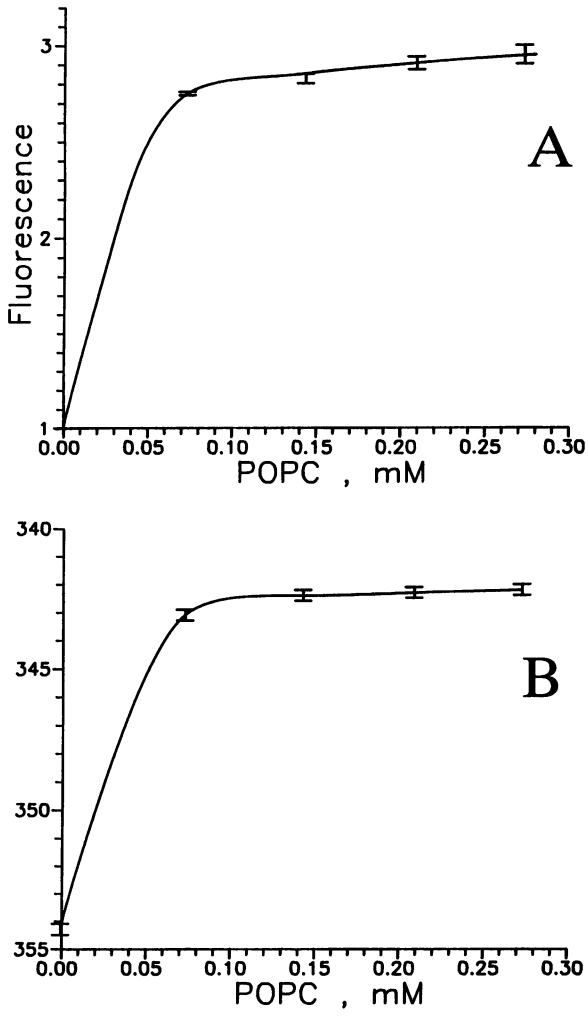

FIGURE 1 Change of TOE fluorescence upon binding to lipid vesicles. The intensity at 340 nm (*A*) and the position of the emission maximum (*B*) are plotted versus the POPC concentration. The concentration of TOE was 2 μM. In other experiments ([POPC] $\gg$ 0.3 mM), practically all of the fluorescence was coming from the membrane-bound TOE.

free TOE. Such data can be analyzed by a variety of mathematical expressions that are designed to quantitate the association of ligand with the membrane in terms of binding and/or partitioning (Blatt et al., 1984; Blatt and Sawyer, 1985). However, in this study our primary interest was not in the binding itself but on the environment of the bound molecule. As, in subsequent experiments, the lipid/probe ratio ranged from 400 to 1000 the fluorescence measured is that of virtually completely membrane-bound TOE. It should be noted that no change in vesicle size was observed after addition of the TOE.

The temperature dependence of the inverse fluorescence intensity of the membrane-bound TOE is presented in Fig. 2. The observed changes are linear for POPC vesicles and sigmoidal for DMPC vesicles, suggesting that TOE fluorescence is sensitive to acyl chain phase transition. With increasing temperature, a moderate red spectral shift was also observed (Fig. 3). The position of the center of weight of the

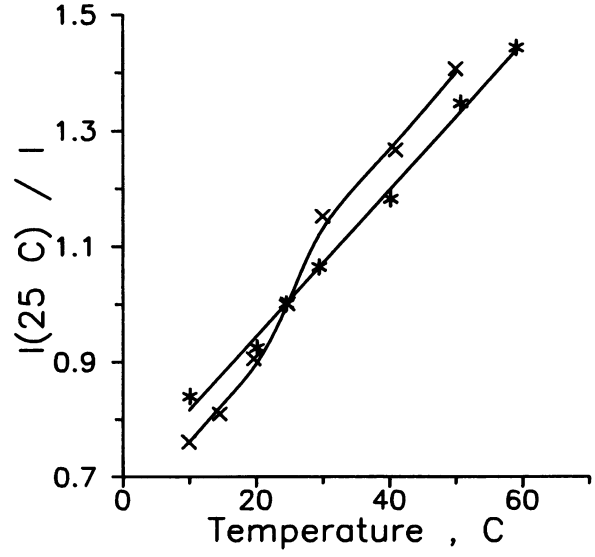

FIGURE 2 Temperature dependence of the reciprocal fluorescence intensity of the TOE bound to POPC (*) and DMPC (×) vesicles. The data are normalized to a value of unity at room temperature. TOE fluorescence is sensitive to the acyl chain phase transition.

spectral distribution was found to depend on the excitation wavelength. This so-called red-edge effect originates from both a variation in the energy of interaction of the fluorophore with its environment on the one hand and from slow (comparable with a fluorescence lifetime) structural dynamics in the excited solvate (defined as the fluorophore and the molecules in its first coordination sphere) on the other

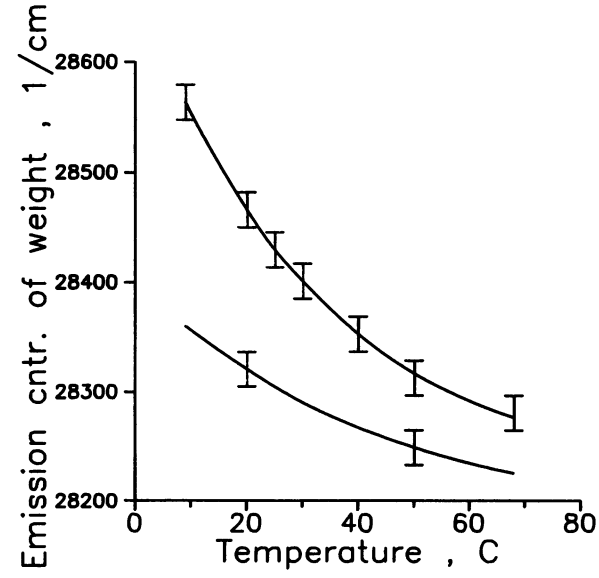

FIGURE 3 Temperature dependence of the position of the center of weight of the emission spectrum of TOE bound to POPC vesicles. Fluorescence was excited either at the center of the absorption band (280 nm; upper curve) or at the red edge (300 nm; lower curve). The temperature-dependent red-edge effect indicates that the environment of membrane-bound TOE has dipolar groups with the nanosecond structural mobility.

(Nemkovich et al., 1992). Structural relaxation occurring on the nanosecond time scale is also consistent with the fact that the decay-associated spectra for membrane-bound TOE corresponding to the longer lifetimes are shifted to the red with respect to those corresponding to shorter lifetimes (Fig. 4).

To determine the depth of penetration of the TOE into the bilayer, vesicles were added that were made entirely from bromolipids with bromines attached to specific positions on the acyl chains. This results in a lower intensity, a shorter average lifetime and a slightly red shifted spectrum as compared with those for TOE in POPC (Figs. 5 and 6 and Table 1). To ensure that the remaining fluorescence does not originate from the unbound TOE, the steady-state anisotropy, r, was measured. In the absence of membranes, TOE fluorescence is practically depolarized (r = 0.004). In POPC, r = 0.06 whereas, for all BRPCs, r is in the range 0.08–0.11, suggesting that this fluorescence originates from membrane-bound TOE. The difference in r between POPC- and BRPC-bound TOE can be attributed to the difference in the excited state lifetime in the two vesicles.

The fluorescence decay of TOE is heterogeneous, and three decay times are required to adequately describe it (Table 1, Fig. 6). When the decay is fitted with only two lifetimes, the residuals and auto-correlation are not distributed randomly and the value for χ^2 is more than two. Quenching results in a decrease in all three lifetimes (which is consistent with a dynamic quenching mechanism), although the contribution from the shortest lifetime increases. The latter could be due to transient effects in diffusion (Lakowicz et al., 1987), which are more pronounced in systems with a high viscosity. The changes in the average lifetime parallel the changes in the steady-state intensity, as expected for dynamic quenching. Additional evidence for a dynamic quenching mechanism comes from the fact that quenching becomes more efficient at higher temperatures

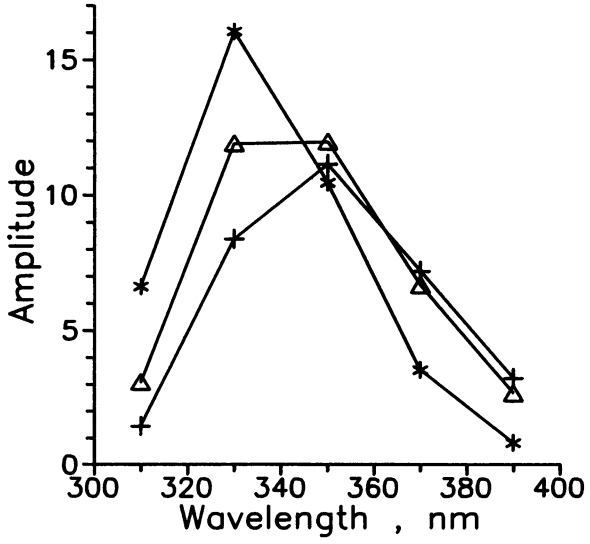

FIGURE 4 Decay-associated spectra of TOE bound to POPC vesicles measured with 285-nm excitation at 20°C. Spectra correspond to the following lifetimes: 0.5 ns (✱); 2.2 ns (△); 5.7 ns (+). Global reduced χ^2 = 1.22. Fluorescence decay is heterogeneous and becomes slower at longer wavelength.

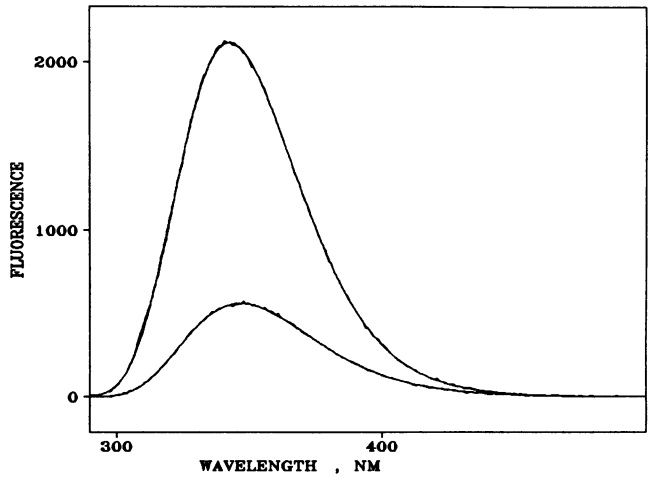

FIGURE 5 Steady-state fluorescence spectra and results of the approximation with the log-normal distribution for TOE bound to vesicles made of quenching (lower curves) and nonquenching lipids (upper curves). Fitted curves practically coincide with experimental data. The following parameters for the log-normal distribution (Eq. 1) were recovered: I_0 = 2120; λ_{max} = 342.2 nm; Γ = 53.0 nm; and ρ = 1.355 for TOE bound to POPC vesicles; and I_0 = 560; λ_{max} = 346.9 nm; Γ = 57.9 nm; and ρ = 1.408 for TOE bound to 9,10BRPC vesicles. The experimental data are well fit by the log-normal approximation. Fluorescence quenching of TOE by bromolipids is accompanied by a red shift and a broadening of the spectrum.

TABLE 1 Fluorescence decay parameters for lipid-bound TOE

TOE + lipid	Decay parameters	τ_a (ns)	Intensity (%)
A. 20°C			
No lipid	τ_1 = 0.17 ns; α_1 = 28%	1.25	40
	τ_2 = 0.88 ns; α_2 = 33%		
	τ_3 = 2.34 ns; α_3 = 39%		
POPC	τ_1 = 0.50 ns; α_1 = 28%	2.90	100
	τ_2 = 2.06 ns; α_2 = 36%		
	τ_3 = 5.64 ns; α_3 = 36%		
6,7BRPC	τ_1 = 0.16 ns; α_1 = 50%	0.87	27
	τ_2 = 0.73 ns; α_2 = 31%		
	τ_3 = 3.00 ns; α_3 = 19%		
9,10BRPC	τ_1 = 0.19 ns; α_1 = 42%	1.14	30
	τ_2 = 0.84 ns; α_2 = 32%		
	τ_3 = 2.99 ns; α_3 = 26%		
11,12BRPC	τ_1 = 0.27 ns; α_1 = 38%	1.31	39
	τ_2 = 1.00 ns; α_2 = 33%		
	τ_3 = 3.03 ns; α_3 = 29%		
DBRPC	τ_1 = 0.20 ns; α_1 = 43%	1.27	33
	τ_2 = 0.82 ns; α_2 = 26%		
	τ_3 = 3.13 ns; α_3 = 31%		
B. 50°C			
POPC	τ_1 = 0.37 ns; α_1 = 25%	1.97	69
	τ_2 = 1.20 ns; α_2 = 25%		
	τ_3 = 3.16 ns; α_3 = 50%		
9,10BRPC	τ_1 = 0.11 ns; α_1 = 43%	0.46	17
	τ_2 = 0.42 ns; α_2 = 35%		
	τ_3 = 1.21 ns; α_3 = 22%		

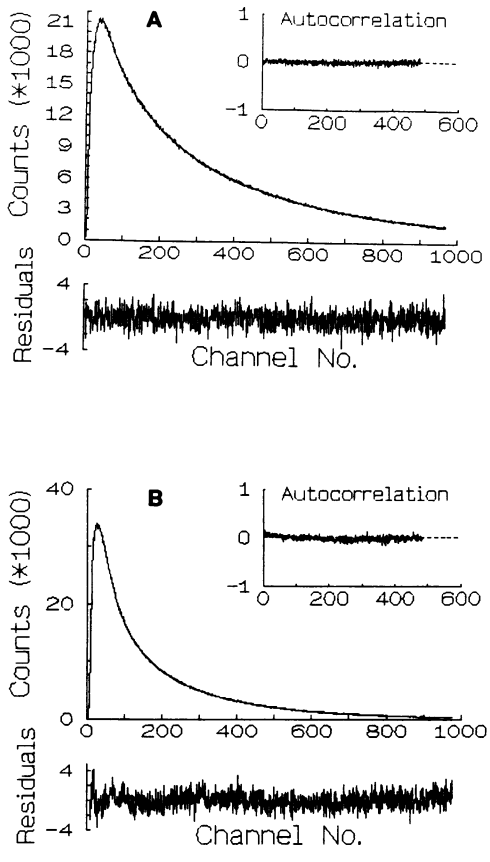

FIGURE 6 Experimental decay curves and the results of a triple exponential analysis for the TOE bound to POPC (*A*) and 9,10BRPC (*B*) vesicles. The timing calibration was 11 ps/channel. Recovered decay parameters are presented in Table 1. Fluorescence quenching of TOE by bromolipids is a dynamic process that reduces the excited state lifetime.

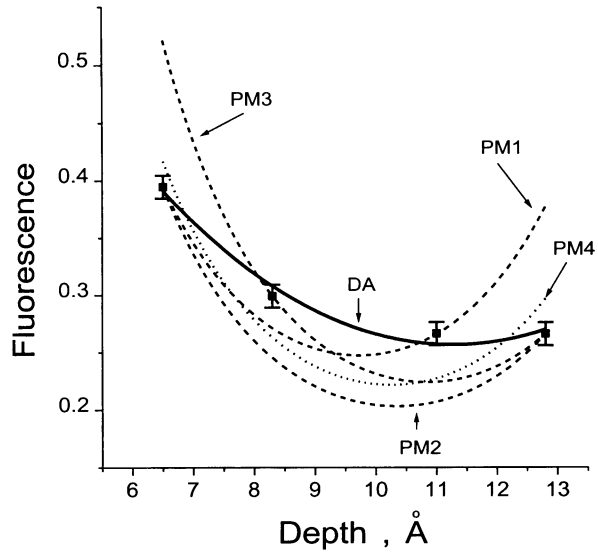

FIGURE 7 Comparison of the DA and PM analysis of the depth-dependent quenching. The fluorescence of TOE in different bromolipids normalized to the intensity in POPC (data from our earlier publication, Ladokhin et al., 1993a) at 20°C plotted versus the average distance from the bilayer center to the bromine atoms (McIntosh and Holloway, 1987). Different pairs of quenchers were used to calculate the PM parameters according to Chattopadhyay and London (1987). Dashed lines correspond to the simulation with Eq. 6 of the result of the PM analysis with the following parameters: h_m = 9.7 Å, R_c = 5.6 Å (PM1); h_m = 10.4 Å, R_c = 6.0 Å (PM2); h_m = 10.8 Å, R_c = 5.8 Å (PM3), calculated from intensities measured with 6,7- and 11,12BRPC; 4,5- and 11,12BRPC; and 4,5- and 9,10BRPC pairs, respectively. Dotted line (PM4) corresponds to the PM best fit nonlinear least-squares analysis of all four data points using Eq 6. with the following parameters: h_m = 10.3 Å, R_c = 5.8 Å. The solid line (DA) corresponds to the DA best fit nonlinear least-squares analysis using Eq 2. with the following parameters: h_m = 11.3 Å, σ = 5.5 Å, S = 18.9. Only DA provides a statistically adequate fit.

(Table 1). A surprising result was observed with quenching by DBRPC. Although this lipid has both chains brominated at the 9,10 positions, the quenching achieved with it was less efficient, although marginally, than that obtained with 9,10BRPC.

To characterize the exact location of TOE in the bilayer, the distribution analysis described earlier (Ladokhin, 1993; Ladokhin et al., 1993a) was applied (see Materials and Methods and Appendix for details). The results of a least-squares fit with Eq. 2 of the earlier reported experimental data for depth-dependent steady-state fluorescence quenching of TOE are shown in Fig. 7. Note that parameters presented in the table may differ slightly from those in preliminary publications. This is mainly because an improved set of depths of quenchers was used in the current study. The values of 12.8, 11.0, 8.3, and 6.5 Å for the distance from the bilayer center to the quenching group were used in the calculations for 4,5-, 6,7-, 9,10-, and 11,12BRPC, respectively. Apparently, an increase in temperature has little effect on the most probable depth and dispersion of the quenching profile of the TOE. In contrast, the parameter S, a measure of the effectiveness of quenching, increases, which is again consistent with a dynamic mechanism.

DISCUSSION

In the following sections we will discuss the strategy of the various fluorescence experiments on membrane proteins. We will first use the results of the model studies on TOE, presented above, to establish the basic principles for and the nature of the information available from the recently introduced method of distribution analysis of the depth-dependent fluorescence quenching, DA (Ladokhin, 1993; Ladokhin et al., 1993a). We will demonstrate then how new information on the structure and dynamics of membrane proteins can be extracted with the DA and other fluorescence techniques when the data on TOE are used as reference points.

Depth-dependent fluorescence quenching

Assumptions for quantitative analysis

We will focus our attention now on the assumptions that are necessarily made to provide a quantitative description for

the depth-dependent fluorescence quenching. We also will discuss how those assumptions are consistent with our knowledge of membrane structure and dynamics.

Typical quenching studies are carried out with lipids labeled at specific positions along their acyl chains with either bromines (Markello et al., 1985; Berkhout et al., 1987; Bolen and Holloway, 1990; De Kroon et al., 1990; Ladokhin et al., 1991, 1992a,b,c, 1993a,b; Gonzalez-Manas et al., 1992, 1993; Tretyachenko-Ladokhina et al., 1993; Rodionova et al., 1995) or spin labels (London and Feigenson, 1981a,b,c; Chattopadhyay and London, 1987; Wardlaw et al., 1987; Yeager and Feigenson, 1990; Chung et al., 1992; Abrams and London, 1992, 1993; Jones and Gierasch, 1994). In such experiments, the lowest fluorescence yield is observed when a particular quencher is at the same depth in the membrane as the tryptophan residue in the protein.

To calculate the actual depth of membrane penetration the quantitative analysis referred to as the parallax method is often used (Chattopadhyay and London, 1987; Abrams and London, 1992, 1993). The basic mathematical expression connecting intensities with (F) and without (F_0) quenching with the vertical distance separating fluorophore and a quencher (z) could be written as the following (Chattopadhyay and London, 1987):

$$F/F_0 = \exp\{\pi(z^2 - R_c^2)C\} \quad \text{for} \quad z \le R_c \qquad (3)$$

$$\text{or} \quad F/F_0 = 1 \quad \text{for} \quad z > R_c,$$

where C is the concentration of quenchers and R_c is a parameter describing the size of the sphere of action, which, in the hard-sphere approximation referred to by the authors, should be equal to the sum of the radii of the fluorophore and quencher. The derivation of this equation is based on three assumptions: a random distribution of quenchers with regard to fluorophores in the plane of the membrane, a static mechanism of quenching, and a uniqueness of depth for the entire population of both quencher and probe.

Although this method has helped in defining the approximate degree of penetration of a number of proteins and fluorescent probes into the bilayer, this technique ignores a basic phenomenon that may lead to a significant misinterpretation of the data, i.e., the existence of a depth distribution of both the fluorophore and the quencher due to both transverse (vertical) fluctuations and conformational heterogeneity. In the more recent paper, Abrams and London (1992) made an attempt to consider the fact that the depth of the fluorophore is not unique. They estimated the error introduced in the refinement of the depth with Eq. 3 by assuming that the fluorophore can occupy a continuous range of depth with equal probability. We believe that a Gaussian distribution, as more physically relevant, should be used instead.

The assumption of the static mechanism for quenching was based on the consideration that lateral diffusion is not fast enough to bring a new lipid quencher from the second coordination sphere to the fluorophore during the excitation lifetime of the tryptophan residue or probe with a similar lifetime (London and Feigenson, 1978). By definition, static quenching does not reduce the lifetime and it usually becomes less efficient with increasing temperature (Lakowicz, 1983, pp. 264–265). However, lipid quenchers are known to reduce the lifetime, even for the relatively short-lived tryptophan fluorescence (Clague et al., 1991; Ladokhin et al., 1992b; Ladokhin and Holloway, 1995). In the present experiments, the contribution of lateral diffusion was eliminated by using vesicles made from 100% quenching lipid. Indeed, the substitution of one quenching molecule with an identical molecule from the second coordination sphere during the excitation lifetime will have no effect. Therefore, other types of motion, such as transverse or rotational, must account for the observed reduction in the lifetime of the TOE (Fig. 6, Table 1).

To further differentiate the aforementioned motions let us compare the quenching caused by the DBRPC and 9,10BRPC (Table 1). In both lipids, the bromines are attached to the same position but in BRPC one acyl chain is not labeled. One would expect that an additional time for rotational diffusion would be required for BRPC to match the quenching ability of DBRPC. In contrast to such expectations, the data show that a shorter (although marginally) average lifetime and a smaller intensity is observed for BRPC rather than for DBRPC. The rationalization for this apparent contradiction comes from the following: (i) vertical (transverse) fluctuations are the main cause of dynamic quenching of tryptophan (and other short-lived probes) in membranes and, (ii) as DBRPC is much heavier than BRPC, its thermal fluctuations are less pronounced and the quenching is less efficient.

In previous studies we reported that the time-resolved quenching of the single tryptophan (W109) in the isolated nonpolar peptide of a mutant cytochrome b_5, which lacks W108 and W112, revealed a peculiar type of heterogeneity (Ladokhin et al., 1992b). It was shown that, whereas the lifetime for a certain population of the fluorophores decreased in the presence of BRPC, the lifetime that the remaining fluorophores exhibited was the same in BRPC as in POPC. This is not the case for TOE (Table 1) or for another protein, melittin (Ladokhin and Holloway, 1995), for which all three lifetimes are decreased. The mutant cytochrome b_5 data could be explained by assuming the existence of a population of molecules where the tryptophan residue is completely buried in the protein interior and inaccessible to the lipid phase. The existence of the multiple conformations of both native and mutant cytochrome b_5 was confirmed in our later studies (Ladokhin et al., 1993b). It can now be concluded that different proteins can have their tryptophan residues exposed to a different extent to the lipid phase and that membrane quenching can uncover this phenomenon.

As was established above, vertical transverse fluctuations of the relative position of the quencher are the main cause of the lifetime reduction for tryptophan fluorescence in membranes. Under these conditions, the Smoluckowsky equation for free diffusion would no longer be applicable, unlike the

cases of the three-dimensional diffusion of water-soluble quencher or two-dimensional lateral diffusion in the membrane. Note that in both later examples the concentration of the quencher is uniformly distributed over all space coordinates, which is not true for depth-dependent quenching. To predict rigorously the changes in fluorescence decay caused by the transverse motion of fluorophore and quencher in membrane one has to solve the equation for restricted diffusion. However, even if this is done, the application of such formalism to the heterogeneous decay exhibited by tryptophan residues seems to have very limited practical value for determining the penetration of proteins into the membrane.

In the current study, a semi-empirical approach suggested earlier was utilized (Ladokhin, 1993; Ladokhin et al., 1993a), which employs a relatively simple mathematical procedure and allows the recovery of parameters with a clear physical meaning from the steady-state fluorescence measurements. This approach is preferable to attempting to use individual lifetimes in any quantitative analysis as this would be futile because of the correlation between α and τ values as well as the nonexponentialities introduced by time-dependent diffusion. Although additional information could be obtained from the use of some form of average lifetimes, we chose not to do so in the present study as it is known that, for systems with red edge shift (such as the current example of membrane-bound TOE, Fig. 3), the complexity of the decay is characterized by nonexponentiality (rather then multiexponentiality (Ladokhin, 1995 and unpublished results)) and therefore the amplitude-averaged lifetime will no longer directly correspond to the steady-state intensity.

Comparison of DA and PM

In the mathematical description for the DA of depth-dependent quenching (Eq. 2), advantage is taken of independent evidence that indicates that the density distribution of the lipid-attached moiety (bromine atom or double bond) can be adequately described by a Gaussian distribution of depth (Wiener and White, 1991). Based on a few considerations described in the Appendix the following equation is suggested (this is Eq. 2 in a more detailed form):

$$\ln\{F(h)/F_0\} = S/(\sigma\sqrt{2\pi})\exp\{-1/2[(h - h_m)/\sigma]^2\} \quad (4)$$

The right-hand side of the equation contains a Gaussian function of the depth coordinate (h) with the following parameters: mean depth (h_m), dispersion (σ), and area (S). Note that Eq. 4 was derived assuming that the concentration for each quencher was the same; otherwise the area parameter will become a function of a concentration and thus a function of depth $S(h) = S_0 \cdot C(h)$. S_0 is the concentration-independent quenching efficiency of a particular fluorophore with a particular quencher. $C(h)$ is a table function of concentrations for each quencher versus its depth. The concentration could be expressed in the mole fraction per surface area for one lipid molecule, which is 70 Å^2 (Lewis and Engelman, 1983). For simplicity only, the case where C is independent of h is considered (Materials and Methods). Indeed, in practice this could be easily achieved by utilizing vesicles formed entirely of brominated lipids. In the case of spin labels, when this would not be practical, the intensities in the left-hand side of Eq. 4 can be corrected before fitting, $F_{corr}(h) = F(h)/\exp(C(h))$.

The equations for the PM (Eq. 3) and for the DA (Eq. 4) can be rewritten in a similar form for comparison:

$$\text{DA: } \ln\{F(h)/F_0\}/C(h) = S_0/(\sigma\sqrt{2\pi})\exp\{-1/2[(h - h_m)/\sigma]^2\} \quad (5)$$

$$\text{PM: } \ln\{F(h)/F_0\}/C(h) = \pi\{(h - h_m)^2 - R_c^2\} \quad (6)$$

The identical left-hand side is a function of the fluorescence corrected for the difference in concentration of different quenchers. The right-hand sides are the depth-dependent profiles of quenching represented by either a Gaussian function (DA) or parabola (PM). Note that the formula for DA has three parameters, whereas that for PM has only two. However, for DA of data presented here, four quenching lipids and one control are used, so our system is statistically over-defined and should allow, in principle, the determination of confidence intervals for parameters. On the other hand, for the PM analysis, it was suggested that two measurements in a quenching lipid are enough (Chattopadhyay and London, 1987). In fact, two measurements are not strictly enough to find a unique solution with the PM, and two solutions always exist.

The application of the DA and PM to the same set of data reported earlier for the TOE (Ladokhin et al., 1993a) is shown on Fig. 7. The parameters recovered by the conventional PM (Chattopadhyay and London, 1987) depend strongly on the choice of quenching pair. The simulation with Eq. 6 of the entire quenching curves (dashed lines) for those results shows significant deviations (ranging up to 30% of intensity) from the other data points. And even when all four data points are used in the PM analysis (dotted line) the fit is rather poor. On the contrary, the DA (solid line) provides an adequate fit to the data, and the need for an extra fitting parameter is statistically justified. The number of data points available on the depth scale is limited to 4–5. With this number of degrees of freedom it might not always be possible to choose between DA and PM judging from the goodness of fit only. However, the example presented above clearly demonstrates that the application of DA can dramatically improve the quality of fit.

We would like to emphasize strongly that we view the advantage of DA not only in the better description of experimental data, but also in a clearer presentation of the physical relevance of its parameters. This enables additional information on the quenching profile (the right-hand side of Eqs. 5 and 6) to be readily obtained from the same data. Let us compare the characteristics of the quenching profile, such as the area under the curve, the maximum, and full width at half height (FWHH), derived for DA and PM (Table 2). For PM, the area

TABLE 2 Comparison of the different parameters resulting from analysis of quenching profiles by DA (Eq. 5) and PM (Eq. 6)

Parameter	DA	PM
Most probable depth	h_m	h_m
Area	S	$R_c^3\ 4\pi/3$
FWHH	$\sigma\sqrt{8\ln 2}$	$R_c\sqrt{8}$
"Height" at h_m	$-S/(\sigma\sqrt{2\pi})$	$-R_c^2\ 2\pi$

and FWHH are coupled to the same parameter, R_c. For the DA, on the contrary, those parameters are independent; the area is related only to S and FWHH only to σ. Indeed, if a different fluorophore and quencher pair with different sizes were used or if the fluctuations in their positions were significantly different, the quenching profile should be broadened to a different extent. On the other hand, if the quenching efficiency is altered either by utilizing different quenchers or by protecting the fluorophore from being completely exposed to the lipid phase, the entire area under the quenching profile should be altered. As these various changes can occur independently, the area and FWHH should be expected to be independent, as they are in the DA.

The minimal intensity observed with the set of different quenchers is sometimes used as a measure of the tryptophan's exposure to the lipid phase (Gonzalez-Manas et al., 1993). Our analysis, however, indicates that the entire area under the quenching prophile should be used instead. The maximal "height" of the distribution (Table 2) is connected to the minimal intensity that could be observed if, by chance, a quencher were placed exactly at h_m. The height at h_m is a derived parameter and for DA it is proportional to the S/σ ratio. Consider two identical fluorophores, one rigidly fixed at a certain position and the other widely distributed in the depth of the bilayer. In the latter case the FWHH (and σ) is much larger. In contrast, as the nonfixed fluorophore spends more time outside its most probable depth, its fluorescence intensity seen in the presence of the quencher located at h_m is higher and consequently the height of the quenching profile is lower. But both fluorophores experience the same quenching efficiency and are completely exposed to lipid. Therefore, it is not the height of the quenching profile at maximum (which is altered as S/σ) but the area (which remains the same as S) that is a measure of the quenchability for this fluorophore. In the other words, one should not consider the observed minimal fluorescence as a measure of fluorophore exposure to the lipid phase. Even for qualitative estimates, for which the full DA is not used, we recommend that a better estimation of relative fluorophore exposure is the sum of all of the intensities measured with different lipid quenchers normalized to the number of qunchers used and to the fluorescence intensity measured in a nonquenching lipid.

Application of the DA to tryptophan fluorescence

The results of the application of the DA to TOE in membranes are presented in Fig. 7 and Table 3. The most probable depth of penetration is approximately 11 Å and decreases slightly with increasing temperature. The width of the distribution is rather large, much larger than that found for tryptophan residues in cytochrome b_5. Note that the σ is a parameter of the quenching profile and is not equal to the dispersion in the actual position of the fluorophore. An additional broadening is introduced by the size of the probe and quencher and the dispersion in the quencher's depth. Following the example of Wiener and White (1991), for illustrative purposes only, we will treat the broadening caused by the finite size of the molecule as a Gaussian function. We will assume also that the dispersion of this function for indole would be σ_{Ind} = 2.5 Å. This will allow the properties of the Gaussian function to be used for the simplification of the mathematics required to estimate the dispersion of the distribution of the center of weight of the fluorophore σ_{cw} from the following: $\sigma^2 = \sigma_{cw}^2 + \sigma_{Br}^2 + \sigma_{Ind}^2$ (Wiener and White, 1991). According to the latter report, the dispersion introduced by the size of the bromines and the fluctuation in their position in the BRPC bilayer is σ_{Br} = 3.5 Å. The σ_{cw} for W109 of mutant cytochrome b_5 is estimated to be 0.9 Å. For the rabbit b_5 the distribution is twice as broad (σ_{cw} = 2.1 Å). This might look like rather small broadening considering the number of tryptophans in the native form (W108, W109, and W112), but existence of an effective energy transfer between them demonstrated by the polarization measurements (Ladokhin et al., 1991) can reduce the number of emitting fluorophores resulting in narrower distribution. Obviously, tryptophan residues in proteins have less conformational freedom than TOE, the σ_{cw} for which changes from 3.4 Å at 20°C to 3.7 Å at 60°C. It is surprising how little effect temperature has on the fluctuation in the position of the TOE. Part of the reason is probably because the distribution is already very wide at 20°C. Although the temperature increase results in a faster displacement of the molecules, it preserves the boundaries of the distribution, which are ultimately limited by the bilayer.

TABLE 3 Results of the application of the DA to model and protein systems

System	h_m (Å)	σ (Å)	S(relative units)*	Reference on experimental data
TOE				Ladokhin et al.,
20°C	11.3	5.5	18.9	1993a
40°C	11.1	5.5	21.8	
60°C	11.0	5.7	24.6	
Cytochrome b_5 at 20°C				Ladokhin et al.,
Rabbit (W108, W109, W112)	11.1	4.8	14.2	1991
Mutant (W109)	10.1	4.4	15.3	
Melittin (W19) at 20°C				Ladokhin and
1-min incubation	12.5	6.2	26.6	Holloway, 1995
90-min incubation	10.9	4.9	21.0	

* As vesicles formed entirely of BRPC's or POPC were utilized in all experiments, no normalization to the quencher concentration was required and S is expressed in dimensionless relative units.

Another explanation involves the very nature of the fluorescence technique, capable of monitoring fluorescent molecules only. If the population of molecules is heterogeneous, then the contribution of those molecules with the higher quantum yield will be overemphasized as compared with their molar concentration. As TOE is so widely distributed in the bilayer section, some molecules will come close to the water molecules penetrating into the defects in the packing of the lipid head groups. As a result, their fluorescence will be quenched progressively with increasing temperature by this alternative mechanism. Quenching by water has a rather high activation energy and therefore more TOE molecules will be quenched at higher temperatures. As the contribution of these molecules to the fluorescence will decrease, the depth-dependent quenching profile will be compressed from the direction that corresponds to the bilayer surface (larger h). This will result in a decreasing h_m (which is observed) and narrowing of σ. The latter will compensate the broadening caused by thermal fluctuations.

The third parameter that describes depth-dependent quenching, S, undergoes the most dramatic changes and increases by 30% over the temperature range investigated. The activation energy (E_a) for the membrane quenching estimated from the Arrhenius dependence $S(1/T) = S(0)\exp(-E_a/RT)$ has a value of 1.3 kcal/mole. As was discussed earlier, the S parameter is related to the ability of a particular quencher to reach a particular fluorophore and to quench its fluorescence. As the quenching mechanism for bromolipid quenching of TOE should be the same as for a tryptophan residue in a protein, the difference in S should be attributed solely to the difference in exposure of the indole ring to the lipid phase. Assuming there is 100% exposure of the TOE to the lipid phase at 20°C, the degree of exposure found for W109 of cytochrome b_5 is found to be 80%.

The establishment of the limiting values for the S and σ parameters for tryptophan residues from the experiments with the TOE has major implications for the protein studies. It has been suggested that some proteins can have several conformations in the membrane. For example, different states of aggregation and orientation were suggested to exist for the bee venom melittin in the bilayer (Vogel and Jahnig, 1986; Talbot et al., 1987; Dempsey, 1990). In our previous studies we reported the complex kinetics of melittin incorporation into the membrane, monitored with the help of depth-dependent quenching of W19 (Ladokhin et al., 1992a, 1993a; Ladokhin and Holloway, 1995). These studies indicated that there is (i) a rapid association with the membrane (time scale of seconds) and (ii) a slow sinking into bilayer, as is seen by an increase in intensity in the case of 4,5BRPC and by a decrease in intensity with other bromolipids (time scale of minutes and hours). The evidence for multiple conformations comes from the fact that the dispersion of the quenching profile is much broader and the area for the quenching profile is larger than those for TOE (Table 3). We suggest that the following sequence of events takes place. Melittin binds to the surface of a membrane but this conformation is metastable. Formation of the stable conformation is slow and results in repositioning of W19 closer to the bilayer center and possibly involves a reorientation across the bilayer and/or aggregation of melittin molecules. Thus, at each point in time, two (at least) main populations exist, both of which could be represented as a Gaussian function of depth. The limited amount of data available on the depth coordinate prevents the resolution of those populations by the DA. Instead, the effective distribution, which in this case would have artificially large σ and S parameters, is recovered.

The complex kinetics observed with lipid quenching of melittin is not related to the binding kinetics itself (i.e., transfer from water to the bilayer) as no changes in fluorescence intensities of melittin were observed within 10 s after addition of nonquenching POPC membranes (Ladokhin et al., 1992a; Ladokhin and Holloway, 1995). The heterogeneity of the membrane-bound melittin was also seen by iodide quenching (Ladokhin et al., 1988; Kaszycki and Wasylewski, 1990; Ladokhin and Holloway, 1995).

Other fluorescence measurements

Once the location of the fluorophore is established, one can characterize the dynamic properties of its environment. However, the polarization measurements often used in such studies are extremely difficult to carry out in scattering systems, and interpretation of their results for membranes is ambiguous (Toptygin and Brand, 1994). Thus we concentrated our effort on other fluorescent parameters such as quantum yield and position of spectrum.

The quantum yield of TOE was found to be sensitive to the acyl chain phase transition (Fig. 2). In Fig. 2, the reciprocal of the quantum yield, $1/Q$, is plotted as a function of the temperature as it had been suggested that, for water-soluble proteins in their native state, $1/Q$ is a linear function of $t(°C)/\eta$, where η is the solvent viscosity (Burstein, 1973). By taking the ratio of the quantum yield of the membrane-bound protein to that of the membrane-bound TOE, the effects of the bilayer can be uncoupled from the effects of the protein on the fluorescence of the tryptophan residue. The significantly different slopes of the temperature dependence of the $Q_{Protein}/Q_{TOE}$ reported for the native and for the mutant cytochrome b_5, which lacks W108 and W112 (Ladokhin et al., 1993b), suggest that different tryptophan residues experience different effective viscosities. This could be because of the different degree to which lipid and protein groups contribute to the tryptophan surroundings. The slope for the mutant with the single tryptophan residue (W109) in the membrane-binding domain is almost three times shallower than that of the native form. This correlates with the DA result discussed earlier, which indicates that W109 has greater exposure to the lipid phase than the average exposure of tryptohan residues in the native b_5 (Table 3).

The spectral position of the emission band is an important parameter for characterizing the polarity and ri-

gidity of a fluorophore's environment. In this study the emission spectrum was fitted to a log-normal distribution, as was suggested by Burstein and co-workers (Burstein, 1973; Burstein et al., 1973). This analysis not only improves the detection of small spectral changes but also allows the recovery of additional information on the width and asymmetry. Quenching by bromolipids results in a red shift of the fluorescence for which the straightforward interpretation would be that the blue fluorophores (those emitting at shorter wavelengths) are quenched more effectively than red. However, in the present instance, quenching is accompanied by the broadening of the spectrum (Fig. 5), which is inconsistent with selective quenching. An alternative explanation for the red shift would involve the effect of bromines on those fluorophores that are not quenched by changing the dielectric properties of their environment. It is possible that both effects are contributing to the spectral shift.

The parameterized description of the spectrum proves to be advantageous in recovering the position of the center of weight, which is difficult to do by the integration of the experimental data because of the contribution of the scattering. This is especially important for the experiments with the red-edge excitation and especially for highly scattering membrane samples. For the membrane-bound TOE, a red shift of fluorescence is observed when the excitation wavelength is changed from the mean of the absorbance band (280 nm) to the red edge (300 nm) (Fig. 3). This so-called red-edge effect arises from the variation in the energies of interaction of the fluorophore with its environment (see review of Nemkovich et al., 1992) and was demonstrated in proteins and membranes (Demchenko and Shcherbatska, 1985; Demchenko and Ladokhin, 1988a,b). In the steady-state domain it can be observed only when the structural relaxation in the excited state occurs on the same time frame as, or is slower than, the depletion of the excited state. Because the red-edge shift diminishes with increasing temperature, this suggests that the dipolar relaxation in the TOE's solvate occurs on the nanosecond time scale (Demchenko and Ladokhin, 1988b). Under such conditions the emission registered at the longer wavelength would be expected to be longer lived than the one registered at the shorter wavelength. This is confirmed by the spectral shifts in the decay-associated spectra (Fig. 4). However, dipolar relaxation alone cannot account for the complexity of the decay. The fact that the TOE molecule, which is completely exposed to lipid, experienced slow structural relaxation provides an important reference point for similar measurements on membrane proteins (Ladokhin et al., 1988, 1991; Kaszycki and Wasylewski, 1990; Chattopadhyay and Rukmini, 1993; Mukherjee and Chattopadhyay, 1994). For water-soluble proteins, on the contrary, exposed tryptophan residues show no red-edge effect (Demchenko et al., 1987; Demchenko and Ladokhin, 1988a,b).

Conclusions

The location of TOE in the membrane was determined by depth-dependent fluorescence quenching utilizing brominated lipids. It was shown that quenching occurs via a dynamic mechanism, with vertical fluctuations playing a central role in the process. The activation energy for the quenching was estimated to be 1.3 kcal/mole.

For quantitative analysis of the depth-dependent quenching, the recently described DA was used (Ladokhin, 1993; Ladokhin et al., 1993a), which extends the conventional PM (Chattopadhyay and London, 1987; Abrams and London, 1992, 1993) to account more realistically for the transbilayer distributions of both probe and quencher. DA provides not only more accurate determination of the most probable depth of the fluorophore but also allows the estimation of other important characteristics, including its conformational heterogeneity and accessibility to the lipid phase.

A number of fluorescence properties of membrane-bound TOE, such as lifetime, quantum yield, anisotropy, spectral distribution, and red-edge shift are reported. These data can provide useful reference points for a variety of steady-state and time-resolved fluorescence data for membrane proteins.

APPENDIX

Method of distribution analysis

In the current study we employed a semi-empirical approach suggested earlier (Ladokhin, 1993; Ladokhin et al., 1993a), which has a relatively simple mathematical procedure and allows the recovery of parameters with a clear physical meaning from the steady-state fluorescence measurements. This method can be formulated in the form of the following hypothesis.

Assume the existence of a sphere of action within which the probability for the membrane-bound fluorophore to collide with the membrane-bound quencher and be converted to the ground state before emission is unity. As the bilayer has an anisotropic organization with only two dimensions (in the plane) that are equivalent to each other, the shape of the "sphere" would not in fact be spherical but will have a preferred direction coinciding with the membrane normal. The same considerations can now be applied as those used in the derivation of the sphere of action for static quenching. We would like to emphasize that, despite a similarity in formalism, this does not imply that quenching in membranes is determined by static quenching. Thus the parameters characterizing sphere would have a different meaning (see Discussion). However, we can apply a similar derivation, because the diffusion of both fluorophore and quencher is limited by the bilayer.

We can now calculate the fraction of fluorophores that do not contain a quencher within their sphere of action and thus will not be quenched. As the system follows the Poisson probability distribution, the probability that the fluorophore will emit is proportional to the exponent of the volume of the sphere (Lakowicz, 1983, p. 273). For simplicity, consider the case where the molar concentration of quenchers in the bilayer is the same for the different quenchers. This is easily achieved by utilizing membranes formed entirely of bromolipids. As the transverse diffusion of the lipid-attached quencher and chromophore is limited, the quenching probability will depend mostly on the vertical separation between them. Independent evidence indicates that the depth distribution of the lipid-bound ligand can be adequately described by a Gaussian distribution (Wiener and White, 1991). Thus the section of the sphere of action with the plane that is normal to the membrane (depth profile) will also be a Gaussian function: $G(z) = S/(\sqrt{2\pi}\sigma)\ \exp\{-1/2[(z - z_m)/\sigma]^2\}$. The position of the maximum (z_m) represents the most probable depth separation of the fluorophore and quencher; the dispersion (σ) will depend on (i) the size of the probe and

quencher and (ii) the thermal fluctuations of both; the area (S) is a function of quenching efficiency and concentration of quenchers. Assuming that the shape of the depth distribution of the quencher does not vary with its most probable depth, then the depth-dependent quenching data can be fitted to the following:

$$F_0/F(h) = \exp\{G(S, \sigma, h - h_m)\},$$

where $F(h)$ is a set of intensities measured as a function of vertical distance from the bilayer center to the quencher (h) and normalized to the concentration of quenchers. To recover three parameters (the most probable position of fluorophore h_m, σ, and S), at least three measurements with the quenchers of known and distinctly different depth are required as well as a control measurement with all nonquenching lipid (F_0).

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