Ionization, Partitioning, and Dynamics of Tryptophan Octyl Ester: Implications for Membrane-Bound Tryptophan Residues

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ABSTRACT The presence of tryptophan residues as intrinsic fluorophores in most proteins makes them an obvious choice for fluorescence spectroscopic analyses of such proteins. Membrane proteins have been reported to have a significantly higher tryptophan content than soluble proteins. The role of tryptophan residues in the structure and function of membrane proteins has attracted a lot of attention. Tryptophan residues in membrane proteins and peptides are believed to be distributed asymmetrically toward the interfacial region. Tryptophan octyl ester (TOE) is an important model for membrane-bound tryptophan residues. We have characterized this molecule as a fluorescent membrane probe in terms of its ionization, partitioning, and motional characteristics in unilamellar vesicles of dioleoylphosphatidylcholine. The ionization property of this molecule in model membranes has been studied by utilizing its pH-dependent fluorescence characteristics. Analysis of pH-dependent fluorescence intensity and emission maximum shows that deprotonation of the α-amino group of TOE occurs with an apparent pK_a of ~7.5 in the membrane. The fluorescence lifetime of membrane-bound TOE also shows pH dependence. The fluorescence lifetimes of TOE have been interpreted by using the rotamer model for the fluorescence decay of tryptophan. Membrane/water partition coefficients of TOE were measured in both its protonated and deprotonated forms. No appreciable difference was found in its partitioning behavior with ionization. Analysis of fluorescence polarization of TOE as a function of pH showed that there is a decrease in polarization with increasing pH, implying more rotational freedom on deprotonation. This is further supported by pH-dependent red edge excitation shift and the apparent rotational correlation time of membrane-bound TOE. TOE should prove useful in monitoring the organization and dynamics of tryptophan residues incorporated into membranes.

INTRODUCTION

The role of tryptophan residues in the structure and function of membrane proteins has recently attracted a lot of attention (Deisenhofer and Michel, 1989a,b; Jacobs and White, 1989; Meers, 1990; Michel and Deisenhofer, 1990; Becker et al., 1991; Chattopadhyay and McNamee, 1991; Weiss et al., 1991; Fonseca et al., 1992; Schiffer et al., 1992; Landolt-Marticorena et al., 1993; Wess et al., 1993; Wimley and White, 1993, 1996; Mukherjee and Chattopadhyay, 1994; White and Wimley, 1994; Woolf and Roux, 1994; Hu and Cross, 1995; Kachel et al., 1995; Reithmeier, 1995; Befort et al., 1996; Ferrer-Montiel et al., 1996; Killian et al., 1996). Membrane proteins have been reported to have a significantly higher tryptophan content than soluble proteins (Schiffer et al., 1992). In addition, it is becoming increasingly evident that tryptophan residues in integral membrane proteins and peptides are not uniformly distributed and that they tend to be localized toward the membrane interface, possibly because they are involved in hydrogen bonding (Ippolito et al., 1990) with the lipid carbonyl groups or interfacial water molecules. The interfacial region in membranes is characterized by unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane (Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992; Slater et al., 1993; Venable et al., 1993; White and Wimley, 1994; Gawrisch et al., 1995; Lewis et al., 1996). The tryptophan residue has a large indole side chain that consists of two fused aromatic rings. In molecular terms, tryptophan is a unique amino acid, because it is capable of both hydrophobic and polar interactions. In fact, the hydrophobicity of tryptophan, measured by partitioning into bulk solvents, has previously been shown to be dependent on the scale chosen (Fauchere, 1985). Tryptophan ranks as one of the most hydrophobic amino acids on the basis of its partitioning into polar solvents such as octanol (Fauchere and Pliska, 1983), whereas scales based on partitioning into nonpolar solvents like cyclohexane (Wolfenden et al., 1979; Radzicka and Wolfenden, 1988) rank it as only intermediate in hydrophobicity. This ambiguity results from the fact that although tryptophan has the polar -NH group which is capable of forming hydrogen bonds, it also has the largest nonpolar accessible surface area among the naturally occurring amino acids (Wimley and White, 1992). Wimley and White (1996) have recently shown from partitioning of model peptides to membrane interfaces that the experimentally determined interfacial hydrophobicity of tryptophan is highest among the naturally occurring amino acid residues, thus accounting for its specific interfacial localization in membrane-bound peptides and proteins. Because of its aromaticity, the tryptophan residue is capable of π-π interactions and of weakly

Received for publication 15 October 1996 and in final form 11 May 1997.

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0006-3495/97/08/839/11 $2.00

polar interactions (Burley and Petsko, 1985, 1988). The amphipathic character of tryptophan gives rise to its hydrogen bonding ability, which could account for its orientation in membrane proteins and its function through long-range electrostatic interaction (Fonseca et al., 1992). The amphipathic character of tryptophan also explains its interfacial localization in membranes due to its tendency to be solubilized in this region of the membrane, besides favorable electrostatic interactions and hydrogen bonding. In addition, tryptophan residues have been implicated in the translocation of membrane proteins (Schiffer et al., 1992) and in the formation of nonbilayer phases due to hydrophobic mismatch (Killian et al., 1996).

The fluorescence of tryptophan and its parent indole has been extensively studied (Beechem and Brand, 1985; Eftink, 1991; Swaminathan et al., 1994; Eftink et al., 1995; Yu et al., 1995). We have recently studied the fluorescence of serotonin, a naturally occurring derivative of tryptophan, which acts as a neurotransmitter in the central and peripheral nervous systems (Chattopadhyay et al., 1996). The presence of tryptophan residues as intrinsic fluorophores in most proteins makes them an obvious choice for fluorescence spectroscopic analyses of such proteins, because in such cases the question of perturbation by an extrinsic fluorophore is eliminated. However, the analysis of fluorescence from multitryptophan proteins is often complicated because of the complexity of fluorescence processes in such systems, and the heterogeneity in fluorescence parameters (such as quantum yield and lifetime) due to environmental sensitivity of individual tryptophans (Chattopadhyay and McNamee, 1991; Eftink, 1991; Mukherjee and Chattopadhyay, 1994). Use of suitable model systems could be helpful in such cases. Despite the importance of membrane-bound tryptophan residues, very few model systems have been developed that could help researchers understand the behavior of tryptophan residues in the membrane. One compound that has previously been used (London and Feigenson, 1981; Jain et al., 1985; London, 1986; Abrams and London, 1992; Ladokhin et al., 1993; Ladokhin and Holloway, 1995) as a simple model for membrane-bound tryptophan (see Fig. 1) is tryptophan octyl ester (TOE). Although in most of these works TOE has been used merely as a marker for membrane-bound tryptophan without detailed characterization, Ladokhin and Holloway (1995) have examined fluorescence from membrane-bound TOE as a model for studying the intrinsic fluorescence of membrane proteins, with special emphasis on the location of the tryptophan moiety in the membrane interior, studied by depth analysis. In this paper we have characterized the behavior of this molecule in the membrane by monitoring its ionization characteristics in model membranes of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and by studying its partitioning behavior and motional characteristics in membranes as a function of its ionization state. Furthermore, the membrane microenvironment experienced by the tryptophan moiety as a function of its ionization status has been characterized by red edge excitation shift (REES) studies and by fluorescence lifetime analyses.

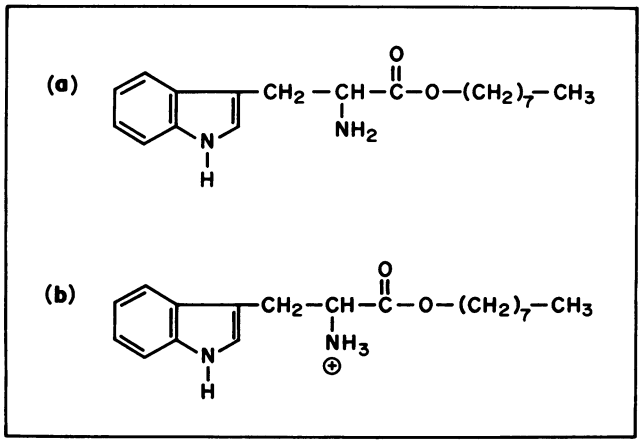

FIGURE 1 Structures of (*a*) deprotonated and (*b*) protonated forms of TOE.

MATERIALS AND METHODS

Materials

DOPC was purchased from Avanti Polar Lipids (Birmingham, AL). Its purity was checked by thin-layer chromatography on silica gel plates in chloroform/methanol/water (65:35:5, v/v/v). It gave one spot with a phosphate-sensitive spray and on subsequent charring (Dittmer and Lester, 1964). Lipid concentration was determined by phosphate assay subsequent to total digestion by perchloric acid (McClare, 1971). Dimyristoyl-*sn*-glycero-3-phosphocholine was used as a standard to assess lipid digestion. TOE was obtained from Sigma Chemical Co. (St. Louis, MO). Its purity was confirmed by thin-layer chromatography on silica gel plates (Abrams and London, 1992) in *n*-hexane/methanol/diethyl ether/acetic acid (80:25:20:1, v/v/v/v), and it gave a single spot with both ninhydrin as well as Ehrlich spray (Stewart and Young, 1984). All other chemicals used were reagent grade. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Methods

Unilamellar vesicles (ULVs) of DOPC containing 1% or 2% (mol/mol) TOE were prepared by the ethanol injection method (Batzri and Korn, 1973; Kremer et al., 1977). In general, 320 nmol of DOPC in chloroform was mixed with 3.2 nmol of TOE in methanol (for experiments in which fluorescence polarization was measured, 1280 nmol of DOPC was mixed with 12.8 nmol of TOE; and for the REES and fluorescence lifetime measurements, 1280 nmol DOPC was mixed with 25.6 nmol TOE). A few drops of chloroform were added and mixed well, and the samples were dried under a stream of nitrogen while warming gently (35°C). After further drying under a high vacuum for 3 h, the dried mixture was dissolved in ethanol, to give a final concentration of ~40 mM lipid in ethanol. This ethanolic solution was then injected into 1.5 ml of the appropriate buffer while vortexing. The buffers used were 10 mM acetate/150 mM NaCl (pH 3–5), 10 mM phosphate/150 mM NaCl (pH 6–8), 10 mM tris-(hydroxymethyl)aminomethane/150 mM NaCl (pH 9), 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)/150 mM NaCl (pH 10) and 50 mM CAPS/150 mM NaCl (pH 11). Background samples were prepared the same way, except that TOE was omitted.

For determination of partition coefficients, ULVs of varying DOPC concentrations were prepared as mentioned above, and small aliquots of

TOE were added from a stock solution of TOE in methanol to the preformed vesicles and mixed well. These samples were then kept in the dark overnight before fluorescence was measured, to ensure complete equilibration.

Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer, using 1-cm path-length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used in all experiments. For single point intensity measurements, the excitation and emission wavelengths used were 280 and 337 nm, respectively. Background intensities of samples in which TOE was omitted were subtracted from all reported values. All spectra were recorded using the correct spectrum mode. Background intensities of samples in which TOE was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. Optical densities and inner filter effects were negligible. All experiments were done with multiple sets of samples; average values of fluorescence and polarization are shown in the figures. The spectral shifts obtained with different sets of samples were identical in most cases. Polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation (Chen and Bowman, 1965)

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. All experiments were done at 25°C. The membrane/water partition coefficient K_p of TOE was determined using the approach developed by Huang and Haugland (1991), which utilizes the enhancement of fluorescence on partitioning of the fluorophore from water into the membrane (see later).

Time-resolved fluorescence measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (17 ± 1 inches of mercury vacuum) and is run at 22–25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox as the scatterer. To optimize the signal-to-noise ratio, 5000 photon counts were collected in the peak channel. All experiments were performed using slits with a nominal bandpass of 8 nm. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. The data stored in a multichannel analyzer was routinely transferred to an IBM PC for analysis. Intensity decay curves so obtained were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i. The decay parameters were recovered using a nonlinear least-squares iterative fitting procedure based on the Marquardt algorithm (Bevington, 1969). The program also includes statistical and plotting subroutine packages (O'Connor and Phillips, 1984). The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals (Lampert et al., 1983), and the autocorrelation function of the weighted residuals (Grinvald and Steinberg, 1974). A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value (not more than 1.6). Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors with the following equation (Lakowicz, 1983):

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (3)$$

Global analysis of lifetimes

The primary goal of the nonlinear least-squares (discrete) analysis of fluorescence intensity decays discussed above is to obtain an accurate and unbiased representation of a single fluorescence decay curve in terms of a set of parameters (i.e., α_i, τ_i). However, this method of analysis does not take advantage of the intrinsic relations that may exist between the individual decay curves obtained under different conditions. A condition in this context refers to temperature, pressure, solvent composition, ionic strength, pH, excitation/emission wavelength, or any other independent variable that can be experimentally manipulated. This advantage can be derived if multiple fluorescence decay curves, acquired under different conditions, are simultaneously analyzed. This is known as the global analysis, in which the simultaneous analyses of multiple decay curves are carried out in terms of internally consistent sets of fitting parameters (Knutson et al., 1983; Beechem, 1989, 1992; Beechem et al., 1991). Global analysis thus turns out to be very useful for the prediction of the manner in which the parameters recovered from a set of separate fluorescence decays vary as a function of an independent variable, and helps distinguish between models proposed to describe a system.

In this paper we have obtained fluorescence decays as a function of pH. The physical model under investigation is of two distinct populations, namely the ionized and un-ionized forms of membrane-bound TOE, which give rise to the observed decay patterns, either as pure components or as mixtures. The global analysis, in this case, thus assumes that the lifetimes are linked among the data files (i.e., the lifetimes for any given component are the same for all decays), but that the corresponding preexponentials are free to vary. This is accomplished by using a matrix mapping of the fitting parameters in which the preexponentials are unique for each decay curve while the lifetimes are mapped out to the same value for each decay. All data files are simultaneously analyzed by the least-squares data analysis method using the Marquardt algorithm (as described above), utilizing the map to substitute parameters appropriately while minimizing the global χ^2. The program used for the global analysis was obtained from Photon Technology International.

RESULTS

The fluorescence characteristics of TOE incorporated into model membranes of DOPC are typical of a membrane-bound tryptophan derivative (Jain et al., 1985; Ladokhin and Holloway, 1995). Tryptophan fluorescence is known to be sensitive to pH (White, 1959; Cowgill, 1963; De Lauder and Wahl, 1970; Ricci, 1970; Jameson and Weber, 1981; Eftink, 1991). We have utilized this property of tryptophan fluorescence to follow the ionization of TOE in membranes. Fig. 2 shows the effect of pH on the fluorescence of TOE incorporated into ULVs of DOPC. As shown in Fig. 2 *a*, there is a steady increase in TOE fluorescence up to pH 10, after being constant up to pH 6. We interpret this change in fluorescence as indicative of the deprotonation of the α-amino group of tryptophan in the membrane (see Fig. 1). In fact, the behavior of TOE fluorescence in this pH range is similar to that of tryptophan in aqueous solution (White, 1959; Cowgill, 1963; Jameson and Weber, 1981; Eftink, 1991). It is well established, from studies of the pH dependence of the fluorescence of tryptophan and its derivatives,

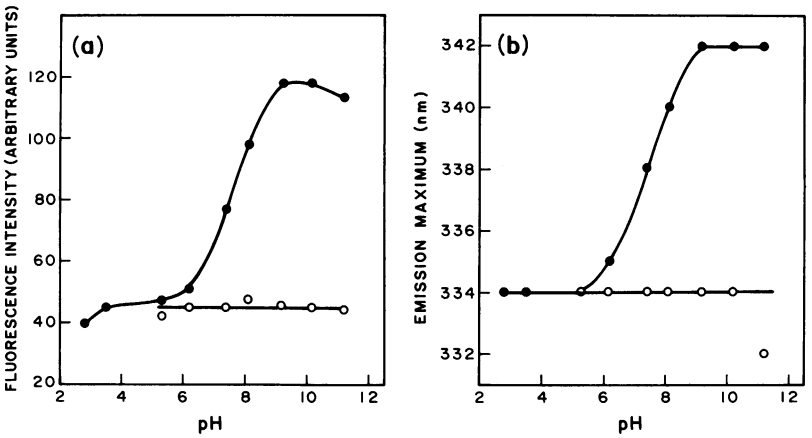

FIGURE 2 Effect of pH on (*a*) the fluorescence intensity and (*b*) fluorescence emission maximum of 1 mol % TOE incorporated into ULVs of DOPC (●). Samples were made by dilution of 320 nmol total lipid in 1.5 ml of buffer by the ethanol injection method. The final ethanol concentration in the samples was 0.5% (v/v). Emission was monitored at 337 nm. The lower lines (○) correspond to fluorescence reversibility upon acidification of high pH samples. pH was lowered by adding different aliquots of 1 M acetic acid, and fluorescence was remeasured immediately. After pH reversal, all of the samples had a pH of 4.0 ± 0.3. Values shown are corrected for dilution upon acidification. See Materials and Methods for other details.

that in compounds with an amino group in the vicinity of the indole ring, fluorescence is more quenched when the amino group is protonated (Beechem and Brand, 1985). The increase in TOE fluorescence with increasing pH can thus be attributed to the release of quenching of TOE fluorescence with deprotonation of the α-amino group at higher pH. Fig. 2 *b* shows the change in fluorescence emission maximum accompanying this pH change. The emission maximum changes from 334 nm to 342 nm when the pH is changed from 4 to 9.

If this change in fluorescence intensity and emission maximum corresponds to deprotonation, it should be reversible. This was tested by the addition of acetic acid to ULVs, permitting fast equilibration of internal and external pH. Fig. 2 (*lower lines*) shows that these fluorescence changes are indeed reversible, thus confirming that the fluorescence change was due to deprotonation. The apparent pK_a value derived from Fig. 2 is ~7.5 for the α-amino group of membrane-bound TOE. (All pK_a values reported are apparent pK_a.) The pK_a values deduced from change in fluorescence intensity and emission maximum are consistent within the range of experimental errors.

In a control experiment, we examined the effect of pH on TOE itself without incorporating it into the membrane, by monitoring its fluorescence as a function of pH in aqueous medium (without lipid). This showed a pK_a of ~9.7. Such a shift of ~2 pH units in pK_a has previously been reported for ionizable groups in molecules when present in aqueous solution and in their membrane-bound form (Kantor and Prestegard, 1978; Ptak et al., 1980; Chattopadhyay and London, 1988).

The membrane/water partition coefficient K_p of TOE was determined using the approach developed by Huang and Haugland (1991), which utilizes the enhancement of fluorescence on partitioning of the fluorophore from water into the membrane. The membrane/water partition coefficient of a probe is defined as

$$K_p = \frac{P_B/L}{P_F/W} \tag{4}$$

where P_B, P_F, L, and W refer to molar concentrations of the membrane-bound probe, the free probe in aqueous phase, the lipid, and water, respectively. Assuming that the quantum yield of fluorescence of the probe increases upon partitioning into the membrane, the experimentally measured fluorescence, F, should be proportional to the concentration of the membrane-bound probe, that is,

$$F = \alpha P_B \tag{5}$$

where α is the proportionality constant. Let P_T be the total probe concentration. Then

$$P_T = P_B + P_F \tag{6}$$

If F_o is the maximum fluorescence resulting from total probe incorporation into the membrane, then

$$F_o = \alpha P_T \tag{7}$$

Substituting for P_B and P_T from Eqs. 5 and 7 into Eq. 6,

$$P_F = (F_o - F)/\alpha \tag{8}$$

Substituting for P_B from Eq. 5 and P_F from Eq. 8 into Eq. 4,

$$K_p = \frac{(F/\alpha)/L}{[(F_o - F)/\alpha]/W} \tag{9}$$

The molar concentration of water, W, in the membrane can be approximated to that of pure water (i.e., 55.6 M) because the fractional volume of the lipid is negligible (Huang and Haugland, 1991). Upon rearrangement of Eq. 9,

$$F = \frac{F_o L K_p}{55.6 + K_p L} \tag{10}$$

or

$$1/F = [55.6/(K_p F_o)]1/L + 1/F_o \tag{11}$$

It is thus evident from Eq. 10 that the fluorescence resulting from titration of liposomes against a constant TOE concentration should exhibit a saturation behavior (Fig. 3). Equation 11 shows that the double-reciprocal plot of the fluores-

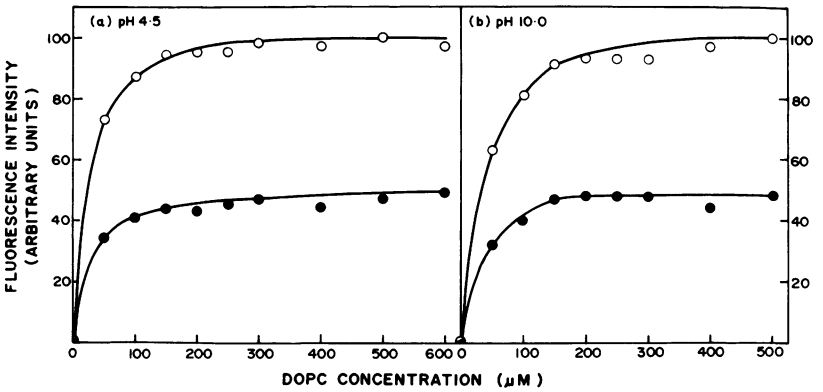

FIGURE 3 Partitioning of TOE into ULVs of DOPC at (*a*) pH 4.5 and (*b*) pH 10. TOE concentration was 1.6 μM (●) or 3.2 μM (○). TOE was added from a methanolic stock solution to preformed ULVs of DOPC and incubated overnight before fluorescence was measured. The emission wavelength was 337 nm. See Materials and Methods for other details.

cence and the lipid concentration should give a linear plot with an x-intercept of $K_p/55.6$ (Fig. 4), from which K_p can be calculated as [55.6 (x-intercept)].

Fig. 3 shows the fluorescence enhancement of TOE on being partitioned into the membrane as it is titrated with varying amounts of ULVs of DOPC at pH 4.5 and 10. In accordance with Eq. 10, this plot shows a saturation profile. It is interesting to note here that the lipid/TOE (mol/mol) ratio at which the curves reach a plateau remains unaltered when the TOE concentration is varied by a factor of 2. It also appears that there is no significant difference between partitioning at pH 4.5 and pH 10 (see later). Fig. 4 shows the corresponding double-reciprocal plots of fluorescence versus DOPC concentration. These plots show good linearity, justifying the use of Eq. 11 for calculating partition coefficients. The common x-intercepts at pH 4.5 and 10 are 3.5×10^4 and 3.6×10^4 M^{-1}, respectively. This corresponds to partition coefficients of 1.9×10^6 and 2.0×10^6 at pH 4.5 and 10. These values of the partition coefficients suggest that TOE can readily partition into the membrane in both its charged and uncharged forms.

The 8-nm shift in TOE fluorescence maximum, in addition to the increase in fluorescence intensity with increasing pH, suggests that the microenvironment of the tryptophan moiety in TOE is changed upon deprotonation. This could mean a change in the membrane environment in the immediate vicinity of the fluorophore. To investigate this possibility, both fluorescence polarization and the red edge excitation shift (REES) of TOE in ULVs of DOPC were monitored as a function of pH. Fig. 5 shows that there is a decrease in polarization with increasing pH from 3 to 11. This indicates more rotational freedom of the fluorophore on deprotonation at higher pH, assuming polarization changes due to self energy transfer to be minimal under these conditions. It is interesting to note that the polarization changes are consistent with the apparent pK_a noted above. Thus these polarization changes are related to the deprotonation of the α-amino group of TOE.

The above results are further supported by changes in REES with pH for membrane-bound TOE. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed the red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases (Demchenko, 1988; Nemkovich et al., 1991; Mukherjee and Chattopadhyay, 1995). This phenomenon arises from the slow rates of solvent relaxation around an excited state fluorophore, which is a function of the mo-

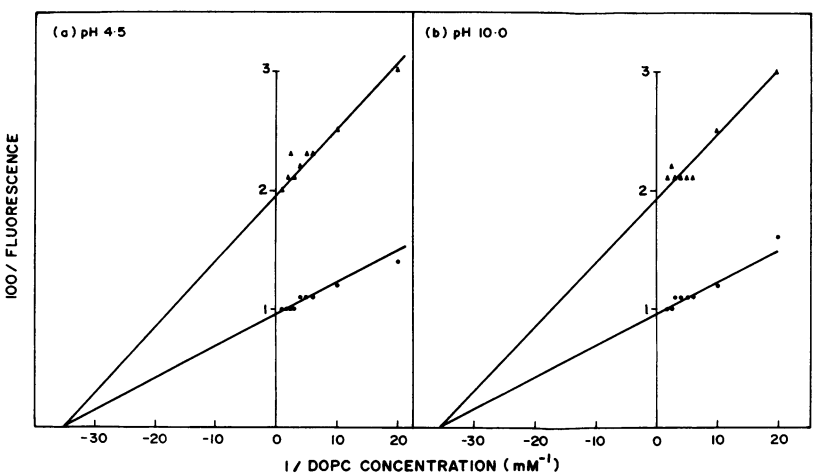

FIGURE 4 Double-reciprocal plots of fluorescence versus lipid concentration for 1.6 μM (▲) and 3.2 μM (●) TOE at (*a*) pH 4.5 and (*b*) pH 10. All other conditions are as in Fig. 3.

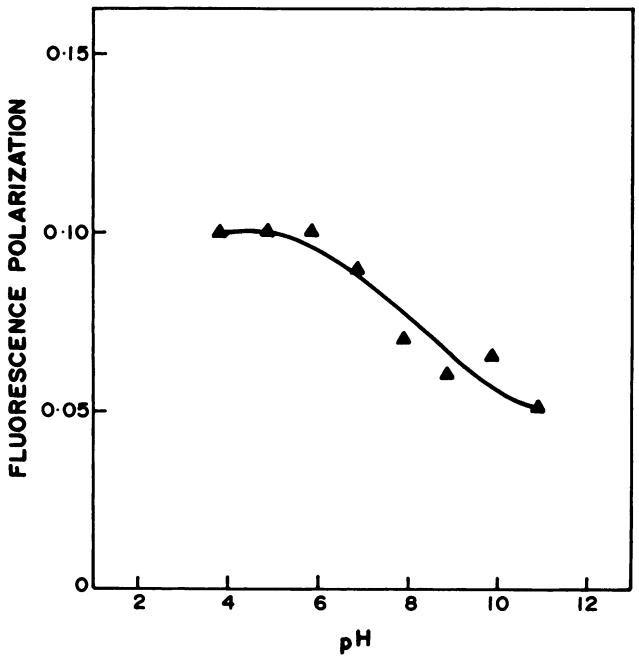

FIGURE 5 Fluorescence polarization of TOE as a function of pH in ULVs of DOPC. The concentration of TOE was 8.5 μM, and the TOE-to-lipid ratio was 1:100 (mol/mol). Samples were excited at 280 nm, and emission was collected at 337 nm. See Materials and Methods for other details.

tional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore (Galley and Purkey, 1970). We have previously shown that REES constitutes a novel and convenient approach for monitoring the organization and dynamics of the microenvironments in which membrane-bound probes or peptides are localized (Chattopadhyay, 1991; Chattopadhyay and Mukherjee, 1993; Chattopadhyay and Rukmini, 1993; Mukherjee and Chattopadhyay, 1994; Guha et al., 1996; Rawat et al., 1997). Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) by using the fluorophore merely as a reporter group. Table 1 shows REES of membrane-bound TOE as a function of pH, when the excitation wavelength is gradually shifted from 280 to 307 nm. The magnitude of REES is 3 nm at pH ~6–7, which decreases to 2 nm at pH 7.8 and finally to 1 nm at pH 8.4 and higher. These results indicate that the tryptophan moiety of TOE is located in a more motionally restricted environment at lower pH values, and the environment becomes more mobile with increasing pH. Taken together, REES and fluorescence polarization results indicate that the trytophan in membrane-bound TOE experiences a restricted environment at low pH and the environment is more dynamic at high pH. This is further supported by changes in apparent rotational correlation time with pH (see later).

TABLE 1 Red edge excitation shifts of TOE in DOPC vesicles as a function of pH*

pH	REES (nm)
6.2	3
7.2	3
7.8	2
8.4	1
9.5	1
11.0	1

*The range of excitation wavelengths used was between 280 nm and 307 nm. All other conditions were as in Fig. 6.

Fluorescence lifetime, which is known to be very sensitive to the microenvironment of the excited-state fluorophore, serves as a sensitive indicator for the ionization state of a fluorophore (De Lauder and Wahl, 1970; Jameson and Weber, 1981; Beddard, 1983; Chattopadhyay et al., 1996). To gain further insight into the pH-dependent changes, we analyzed fluorescence lifetimes of TOE in DOPC vesicles as a function of pH. Table 2 shows the variation of fluorescence lifetime of TOE in DOPC vesicles with pH. All fluorescence decays of membrane-bound TOE could be fitted well with a biexponential function. A typical decay profile, with its biexponential fitting, and the various statistical parameters used to check the goodness of the fit are shown in Fig. 6. As can be seen from the table, for TOE in DOPC vesicles at pH 5.02, the decay fitted a biexponential function with the major lifetime component (preexponential factor 0.96) with a very short lifetime of 0.59 ns, and a minor component (preexponential factor 0.04) with a relatively longer lifetime of 3.74 ns. At higher pH values, there is an increase in the longer lifetime, resulting in an increase in mean fluorescence lifetimes (see Fig. 7). The same set of fluorescence decays was subjected to global analysis. The decays were all assumed to be biexponential (on the basis of the results from discrete analysis), with fixed lifetime components whose relative contributions (preexponential factors) were allowed to vary. The results of the global analysis are shown in parentheses in Table 2. The global normalized χ^2 value obtained was 1.62.

The mean fluorescence lifetimes of membrane-bound TOE were calculated using Eq. 3 and are plotted as a function of pH in Fig. 7 for both discrete and global analysis. It is apparent from the figure that the mean fluorescence lifetime of TOE exhibits a steady increase with increasing pH, irrespective of the method of analysis (discrete or global). We interpret this result to signify that the tryptophan moiety of TOE experiences progressively different microenvironments with changing pH. This could signify a change in hydration of the tryptophan moiety with increasing pH, because tryptophan lifetimes are reduced with an increased water content of the surrounding medium (Kirby and Steiner, 1970; Ho and Stubbs, 1992).

The apparent (average) rotational correlation times for membrane-bound TOE were calculated using Perrin's equation (Lakowicz, 1983):

$$\tau_c = \frac{\langle \tau \rangle r}{r_o - r} \tag{12}$$

TABLE 2 Lifetimes of TOE in DOPC vesicles as a function of pH*

pH	α_1	τ_1 (ns)	α_2	τ_2 (ns)
5.02	0.96 (0.96)#	0.59 (0.59)	0.04 (0.04)	3.74 (3.88)
6.10	0.96 (0.96)	0.59 (0.59)	0.04 (0.04)	4.06 (3.88)
7.00	0.95 (0.95)	0.59 (0.59)	0.05 (0.05)	3.91 (3.88)
8.10	0.95 (0.95)	0.62 (0.59)	0.05 (0.05)	4.18 (3.88)
9.00	0.95 (0.95)	0.61 (0.59)	0.05 (0.05)	4.17 (3.88)
10.08	0.95 (0.94)	0.62 (0.59)	0.05 (0.06)	4.39 (3.88)
11.04	0.95 (0.94)	0.62 (0.59)	0.05 (0.06)	4.43 (3.88)

*Excitation wavelength 296 nm, emission wavelength 337 nm. All other conditions were as in Fig. 6.
#Numbers in parentheses indicate values for global analysis.

where r_0 is the limitimg anisotropy of tryptophan (Weber, 1960), r is the steady-state anisotropy (derived from the polarization values using $r = 2P/(3 - P)$), and $\langle\tau\rangle$ is the mean fluorescence lifetime as calculated from Eq. 3. The values of the apparent rotational correlation times, calculated this way, are shown in Fig. 8. There is a steady decrease in rotational correlation time with increasing pH. This reinforces our earlier conclusion that the trytophan in membrane-bound TOE experiences a restricted environment at low pH and the environment becomes more dynamic with increasing pH.

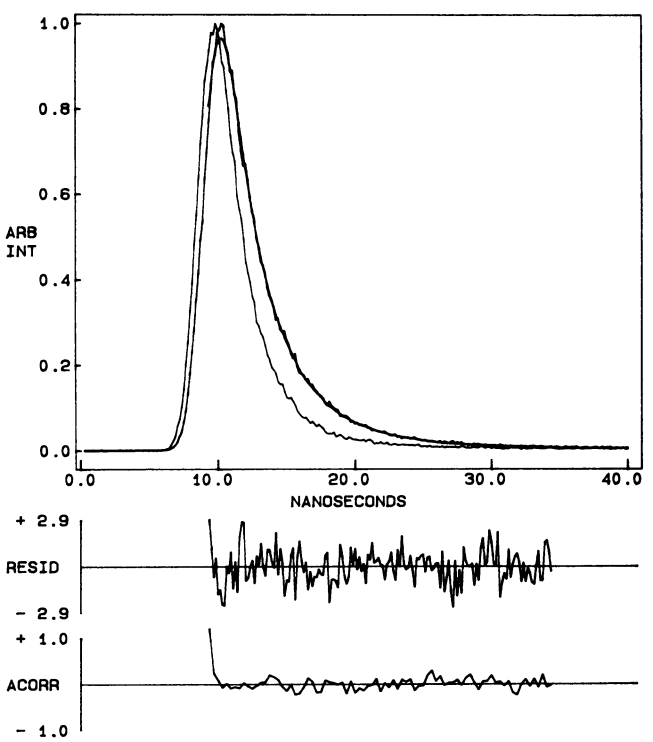

FIGURE 6 Time-resolved fluorescence intensity decay of TOE in ULVs of DOPC at pH 7.0. Excitation was at 296 nm, which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 337 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, fitted to a biexponential function. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. The concentration of TOE was 17.1 μM, and the TOE-to-lipid ratio was 1:50 (mol/mol). See Materials and Methods for other details.

DISCUSSION

Tryptophan residues serve as intrinsic, site-specific fluorescence probes for protein structure and dynamics (Eftink, 1991). Because of the presence of relatively few tryptophan residues in a typical protein, coupled with the fact that its fluorescence properties are responsive to its environment, tryptophans are the most useful intrinsic probes in proteins. Tryptophan fluorescence provides a fairly specific and sometimes very sensitive indication of protein structure and its interactions. In the case of integral membrane proteins, tryptophan residues have been suggested to play an important role in anchoring membrane proteins at precise locations in the bilayer (Schiffer et al., 1992). This may be achieved by proper positioning of the transmembrane helix with respect to the bilayer, because the interfacially localized tryptophan residues could act as "floats," with their polar atoms facing water and the nonpolar part dipped in the lipid bilayer, thereby stabilizing the helix with respect to the membrane environment for optimal activity (Landolt-Marticorena et al., 1993).

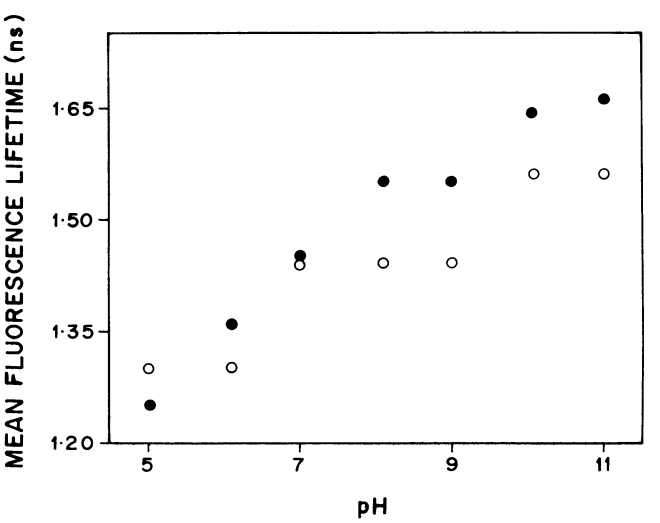

FIGURE 7 Mean fluorescence lifetimes of TOE as a function of pH in ULVs of DOPC obtained by discrete lifetime analysis (●) and global lifetime analysis (○). The excitation wavelength used was 296 nm, and emission was set at 337 nm. Mean lifetimes were calculated from Table 2, using Eq. 3. All other conditions are as in Fig. 6. See Materials and Methods for other details.

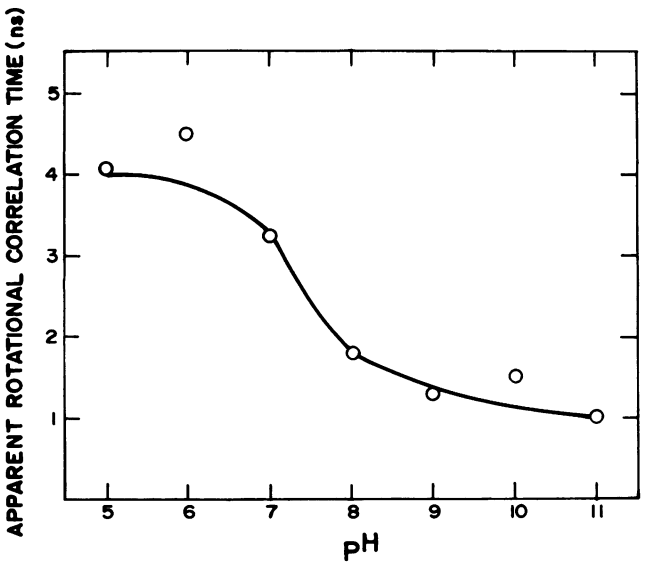

FIGURE 8 Apparent rotational correlation times of TOE as a function of pH in ULVs of DOPC. All other conditions are as in Fig. 7. See text for other details.

The focus of this report is the characterization of TOE as a fluorescent membrane probe, especially with regard to its ionization, partitioning, and motional properties. The ionization characteristics of L-tryptophan in aqueous solution have previously been studied (White, 1959; Cowgill, 1963; De Lauder and Wahl, 1970; Ricci, 1970; Jameson and Weber, 1981; Eftink, 1991). The pH-dependent fluorescence of tryptophan is attributed to the dissociation of both its α-carboxylic acid and α-amino group with approximate pK_a values of 2.6 and 9.2, respectively (De Lauder and Wahl, 1970; Jameson and Weber, 1981; Eftink, 1991). Because the free carboxylic group in TOE is esterified to form the octyl ester, we attribute the pH-dependent changes in its fluorescence solely to the deprotonation of its α-amino group. In this study, we find the behavior of membrane-incorporated TOE to be quite similar to that of tryptophan in aqueous medium, except that the pK_a for the α-amino group is about 1.7 units lower than in aqueous solution. Such shifts (1.5–3 pH units) of pK_a have previously been observed for the carboxyl groups of free fatty acids (Kantor and Prestegard, 1978; Ptak et al., 1980) and 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled phospholipids (Chattopadhyay and London, 1988) incorporated into model membranes. Thus our result is consistent with previous findings on pK_a shifts for membrane-bound probes. It has previously been shown by membrane penetration depth analysis using the parallax method (Chattopadhyay and London, 1987), which utilizes fluorescence quenching by spin-labeled phospholipids, that the tryptophan moiety in TOE is localized in an interfacial region in the membrane (Abrams and London, 1992, 1993). That there is a pK_a shift in membrane-bound TOE relative to that in water is not surprising, as the polarity of this region is distinctly different from that of bulk water (Ashcroft et al., 1981; Perochon et al., 1992).

Our results show that the pK_a of the α-amino group of TOE is ~7.5 when bound to membranes. This would mean that at pH ~7, the tryptophan moiety of TOE exists in membranes as a mixture of protonated and deprotonated forms. Such knowledge of pK_a should help in selecting a suitable pH for experiments in which, at a given pH, a homogeneous population is required to avoid ground-state heterogeneity.

In addition, we have determined the membrane/water partition coefficients for the charged and uncharged forms of TOE into DOPC vesicles by utilizing the fluorescence enhancement of TOE upon partitioning from the aqueous to the membrane phase. The overall charge of a molecule could be important for partitioning of a probe into the membrane. For example, the amphiphilic amine chlorpromazine partitions more strongly into the membrane in its uncharged form than in its charged form (Welti et al., 1984). From our results it appears that the partitioning of TOE into the membrane is only slightly more favored in its uncharged form than its charged form. Because the difference in the partition coefficients is very small (1.9×10^6 and 2.0×10^6 at pH 4.5 and 10, respectively), it is difficult to ascertain whether this represents a true electrostatic effect. This could be attributed to the fact that the major driving force for partitioning of TOE is the hydrophobic effect generated by the incorporation of the octyl tail into the membrane (along with the nonpolar surface of the tryptophan ring), which is independent of the charge of the molecule.

The change in fluorescence polarization of TOE with pH (Fig. 5) could be related to a change in membrane environment upon deprotonation. The decrease in polarization at higher pH is probably related to lack of charge interaction, which eliminates rotational constraints. Furthermore, we have utilized REES as well as rotational correlation times of TOE in DOPC vesicles as a function of pH to monitor tryptophan immobilization in the membrane. Our results show a decrease in the extent of REES and rotational correlation time with increasing pH, implying that the tryptophan environment is more restricted at low pH. It has recently been shown by one of us that for anthroyloxy-labeled fatty acids incorporated into membranes, ionization could bring about a change in their location in the membrane (Abrams et al., 1992), as measured by the parallax method, utilizing fluorescence quenching by spin-labeled phospholipids (Chattopadhyay and London, 1987). The protonation of the carboxyl group resulted in a deeper localization of the anthroyloxy moiety in membrane-bound anthroyloxy-labeled fatty acids. In the case of TOE, however, no significant difference in membrane penetration depth could be detected with a change in ionization state (data not shown).

The fluorescence decay of tryptophan in water is biexponential. This has been attributed to the rotamer model of tryptophan fluorescence, originally proposed by Szabo and Rayner (1980) and recently confirmed by analysis of conformational heterogeneity of tryptophan in crystals of erabutoxin b (Dahms et al., 1995). According to this model,

the lifetime heterogeneity of tryptophan could be attributed to tryptophan rotamers that interconvert slowly on a nanosecond time scale. These rotamers, defined relative to their C_α-C_β bond, have different distances between the carboxylate and amino groups and the indole ring and, consequently, exhibit different extents of electrostatic interactions between these groups. Because the fluorescence lifetime of indole is sensitive to the surrounding environment, different rotamers exhibit different lifetimes. The observation of short lifetimes is generally taken as an indication of an extensive deactivation mechanism. Our results show biexponential decays and a predominant short lifetime component at all pH values (see Table 2). This is in sharp contrast to results obtained with tryptophan in aqueous solutions, where the decay is found to be monoexponential at pH 11 with a long lifetime of 9.1 ns (Robbins et al., 1980). We suggest that in the case of TOE, because of the esterification of the free carboxylic group of tryptophan by the octyl chain (which causes steric crowding), and the fact that the high hydrophobicity of this chain demands that it be embedded in the lipid bilayer, this tryptophan derivative in the membrane environment loses its freedom of rotation about the C_α-C_β bond. This is illustrated in Fig. 9, where the Newman projections of three rotamers along the C_α-C_β bond of TOE in its protonated form are shown. We suggest that rotamer I will be preferred because of 1) lack of steric constraints between the octyl chain of TOE and the indole ring, and 2) the stabilization gained from the energetically favorable electrostatic interaction between the delocalized π electron cloud of the indole ring and the positively charged quarternary nitrogen atom. We also propose that the short lifetime component (which is present at all pH values but predominates at lower pH) corresponds to rotamer I. Upon deprotonation of TOE at higher pH, rotamer I could be somewhat less populated (because of loss of the positive charge of the nitrogen atom, which was responsible for favorable charge interaction with the indole ring), as is apparent from the slight increase in the short-lifetime component with a concomitant decrease in its preexponential factor (Table 2), which results in a higher mean lifetime with increasing pH (Fig. 7). This is accompanied by an increase in fluorescence due to reduced quenching by the uncharged amino group (Beechem and Brand, 1985), as discussed above (see Results). Nevertheless, the predominance of the short-lifetime component, even at high pH, indicates that rotamer I makes a substantial contribution under such conditions because of energetically unfavorable steric crowding in rotamers II and III. In other words, addition of the hydrophobic octyl chain to the tryptophan results in the "freezing" in space of one of the rotamers (rotamer I). Similar frozen rotamers are also expected in the case of tryptophans that anchor transmembrane stretches of amino acids to the membrane interface, the hydrophobic transmembrane amino acid sequences acting as the orienting force in these cases. We must emphasize here that the model proposed here is a plausible one, and alternative interpretations of our data cannot be ruled out at present.

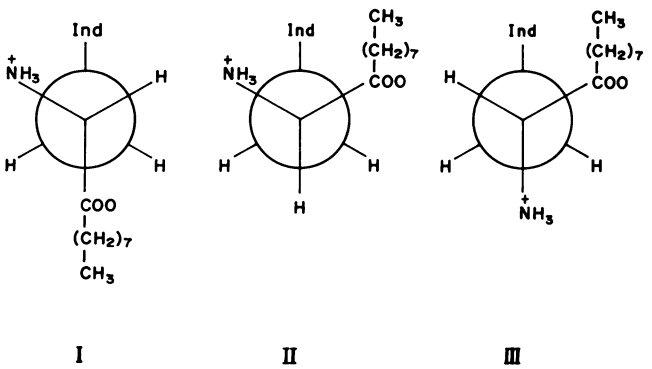

FIGURE 9 Newman projections of three rotamers along the C_α-C_β bond of TOE in its protonated form. Ind is the indole ring of tryptophan. See text for details.

In accordance with the above proposition, we find that the predominance of the short-lifetime component for membrane-bound tryptophan is not restricted to TOE alone. We have observed earlier such short lifetimes in the case of membrane-bound peptides such as melittin, which is a hemolytic peptide (A. K. Ghosh et al., manuscript submitted for publication), and in the channel-forming peptide gramicidin (Mukherjee and Chattopadhyay, 1994). Short lifetimes have also been reported for membrane-bound model α-aminoisobutryl peptides containing tryptophans at various positions (Vogel et al., 1988). We attribute this to the predominance of one of the rotamers around the C_α-C_β bond of tryptophan in these membrane-bound molecules when the tryptophan is oriented at the membrane interface, which is an anisotropic and motionally restricted region (Perochon et al., 1992; White and Wimley, 1994). This arrangement results in one rotamer population being significantly populated, which results in shorter decay times.

This study, along with previous work on TOE fluorescence in membranes (Ladokhin and Holloway, 1995), shows that TOE can be effectively used as a simple model for tryptophan residues in membranes. Knowledge of its ionization and partitioning behavior should help in choosing optimal conditions for its use in membrane studies. The importance of membrane-bound tryptophan residues has already been outlined. TOE should prove useful in monitoring the organization and dynamics of tryptophan residues incorporated into membranes.

We thank Dr. R. Nagaraj for helpful discussions and Dr. G. Krishnamoorthy, Y. S. S. V. Prasad, and G. G. Kingi for their technical help. We are grateful to anonymous reviewers for helpful suggestions.

This work was supported by the Council of Scientific and Industrial Research, and by a grant (SP/SO/D-31/93) from the Department of Science and Technology, Government of India, to AC. SM thanks the University Grants Commission for the award of a Senior Research Fellowship. SS was awarded a Summer Training Program Fellowship by the Centre for Cellular and Molecular Biology. SSR thanks the Council of Scientific and Industrial Research for the award of a Junior Research Fellowship.

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