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Distribution Analysis of Membrane Penetration of Proteins by Depth-Dependent Fluorescence Quenching

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A new approach is presented to evaluate the depth-dependent quenching of the fluorescence of membrane-bound probes and integral proteins. By utilizing at least three quenchers of known and distinctly different depths, the following parameters can be recovered: most probable depth of the probe; dispersion of the depth distribution, which will depend on the size of probe and fluctuations in its position; and quenching efficiency, which is related to the exposure of a particular fluorophore to the lipid phase. The exposure of tryptophan residues in integral proteins can be quantitatively determined with respect to the model compound (tryptophan octyl ester). The proposed method was applied to the investigation of membrane complexes of the bee venom melittin and cytochrome b_5.

KEY WORDS: Membrane penetration; proteins; distribution analysis; depth-dependent quenching.

INTRODUCTION

Many methods of protein structure analysis developed for water-soluble systems are inapplicable to membranes, and there has been increasing use of fluorescence techniques. One of the recent approaches in this area involves the use of lipids labeled at certain positions along their acyl chains with fluorescence quenchers (bromines [1,2] or spin labels [3]). Quantitative analysis used in most of these studies [4,5] is based on three assumptions: a random distribution of fluorophores and quenchers 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 such methods have helped in defining the approximate degree of penetration of a number of proteins and fluorescent probes into the bilayer, these techniques ignore a basic phenomenon, which may lead to a significant misinterpretation of the data: the existence of a depth distribution of both the fluorophore and the quencher due to both transverse (vertical) fluctuations and conformational heterogeneity. The "distribution analysis" (DA) technique proposed here is based on the independent knowledge that quenchers are distributed in the depth of the membrane [6]. DA not only provides the most probable depth of the fluorophore, but also allows the estimation of its conformational heterogeneity and accessibility to the lipid phase.

MATERIALS AND METHODS

Since most of this study is devoted to a reevaluation of earlier published data, the original papers should be consulted for the experimental details on cytochrome b_5 [7], tryptophan octyl ester (TOE) [8], and melittin [9,10]. In studies with melittin the buffer also contained 10 mM EDTA.

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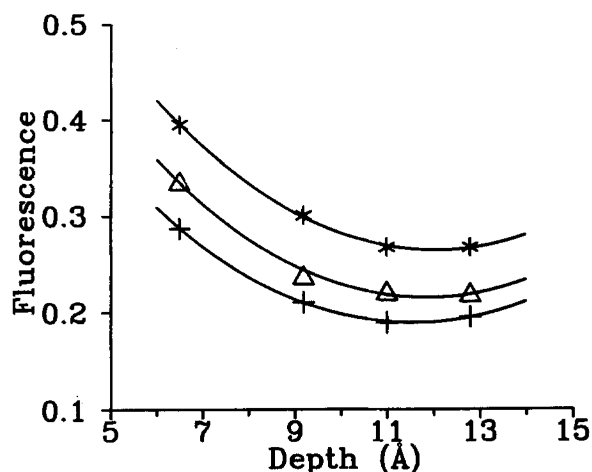

Fig. 1. Relative fluorescence of TOE in different bromolipids plotted versus the average distance from the bilayer center to the bromine atoms [13]. Data for different temperatures are separately normalized to the intensity in POPC. Solid lines correspond to the best-fit nonlinear least-squares analysis using Eq. (1) with the following parameters: h_m = 12.1 Å, σ = 6.5 Å, and S = 39 for 20°C (*); h_m = 11.9 Å, σ = 6.5 Å, and S = 45 for 40°C (△); and h_m = 11.6 Å, σ = 6.6 Å, and S = 49 for 60°C (+).

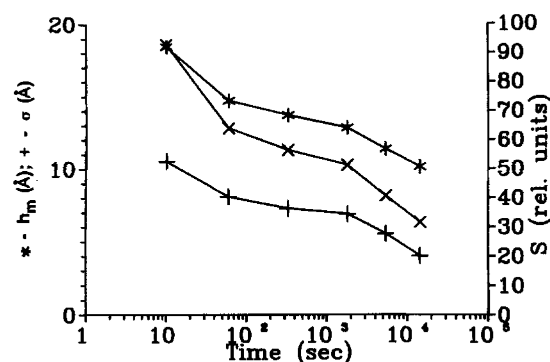

Fig. 2. Kinetics of melittin binding to membrane. Parameters of the depth-dependent quenching of the fluorescence of Trp19 are plotted versus incubation time.

Table I. Parameters of Membrane Penetration of Tryptophan Residues of Cytochrome b_5[a]

Fluorophore	h_m (Å)	σ (Å)	σ_{cw} (Å)	S	Relative exposure (%)
Trp108	13.0	5.2	2.7	20	52
Trp109	10.2	4.8	1.8	28	73

[a]Raw data were published elsewhere [7].

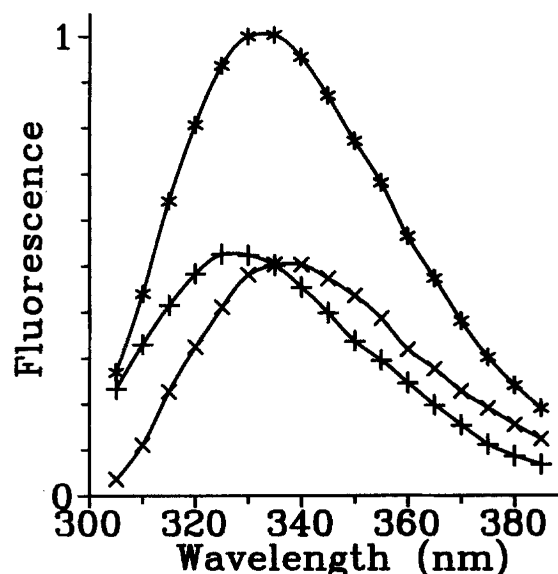

Fig. 3. Quenching-associated steady-state fluorescence spectra for the iodide quenching of membrane-bound melittin: total spectrum (*); spectra associated with K_{SV} = 0.46 M^{-1} (+) and K_{SV} = 7.5 M^{-1} (×).

RESULTS

In the current study we employed a semiempirical approach suggested earlier [11], 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 of the membrane-bound fluorohore colliding with the membrane bound quencher and being converted to the ground state prior to emission is unity. The probability that the fluorophore will emit is proportional to the exponent of the volume of the sphere. For simplicity let us consider the case when 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. Since 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 distribtion [6]. 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: $G(z) = S/(\sigma\sqrt{2\pi})\exp\{-1/2[z - z_m)/\sigma]^2\}$. 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; and the area (S) is a function of the 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:

$$F_0/F(h) = \exp\{G(S,\sigma,h\text{-}h_m)\} \quad (1)$$

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

APPLICATIONS

TOE. The results of the distribution analysis of TOE penetration are shown in Fig. 1. Apparently an increase in temperature has little effect on the most probable depth and dispersion of the quenching profile. On the contrary, the parameter S, a measure of the effectiveness of quenching, increases, which is consistent with a dynamic mechanism.

Cytochrome b_5. Two neighboring tryptophans are shown to penetrate differently into the membrane (Table I). The average depth differs by almost 3 Å and the relative exposure to the lipid phase (relative to that of the TOE) varies from ½ to ¾. Although the difference in σ does not seem to be significant, it is mainly because of the broadening introduced by the size of the probe and quencher and the dispersion in the quencher depth. Utilizing the approach suggested by Wiener and White [6] and assuming the value of 3 Å for the broadening introduced by the size of the indole ring, we calculated the dispersion in the depth of the center of weight σ_{cw}, which appears to be quite different for the two tryptophanyls. Certainly both tryptophanyls have less conformational freedom than TOE.

Melittin. The bee venom peptide melittin, containing a single fluorophore, Trp19, is known to have different conformations in membranes [12]. Earlier we reported the slow dynamics of melittin penetration into the bilayer [10]. The result of reevaluation of these data with the DA is shown in Fig. 2. All three parameters exhibit complex kinetics, which is probably due to transitions between different populations with different depths of penetration. The existence of such populations is revealed by the iodide quenching as well [9]. Figure 3 shows the decomposition of the fluorescence spectra of membrane-bound melittin on components associated with different Stern–Volmer constants. Additional evidence for multiple conformations comes from the fact that the dispersion of the quenching profile in Fig. 2 is much broader than the one for TOE. It should be emphasized that the kinetics observed with lipid quenching is not related to the binding kinetics, as no changes in fluorescence intensities of melittin were observed in 10 s after the addition of POPC membranes [10].

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