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# Fluorescence quenching at interfaces and the permeation of acrylamide and iodide across phospholipid bilayers

Fernando Moro, Félix M. Goñi*, M. Angeles Urbaneja

*Department of Biochemistry, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain*

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Studies of fluorescence quenching in membrane proteins are complicated by the fact that the barrier effect of the bilayer towards the quenchers is not known with precision. Our studies show that (a) both acrylamide and iodide can permeate the membrane at comparable rates, (b) when quenchers are added externally to a vesicle suspension, the apparent Stern–Volmer quenching constants for the same fluorophores are lower in the inner than in the outer aqueous compartments, and (c) at least some non-polar fluorophores embedded in the bilayer are quenched by iodide, but not by acrylamide.

Fluorescence quenching; Lipid bilayer; Tryptophan

## 1. INTRODUCTION

The intrinsic fluorescence of tryptophanyl residues is often used in protein structural studies. In particular, the phenomenon of fluorescence quenching has found widespread application since it is believed to reflect the accessibility of some parts of the polypeptide, i.e. the fluorescent residues, to the quenching molecule. Of course, changes in protein conformation may induce changes in tryptophanyl accessibility, hence the interest of fluorescence quenching studies [1]. This technique has been applied mainly to soluble proteins, however, it is potentially interesting also for membrane-bound proteins, and it has indeed been used in such cases as well [2–5]. Nevertheless, the presence of the lipid bilayer phase complicates the interpretation of the experimental data, since quenchers are usually polar compounds, and their access to the fluorophores may be limited by the quencher solubility in the membrane matrix.

The present paper intends to clarify some of the problems posed by the presence of the lipid bilayer in protein fluorescence quenching measurements, namely the ability of water-soluble quenchers to pass *across* the membrane, and their capacity to act on fluorophores *inside* the bilayer. For this purpose we have carried out direct measurements of the diffusion of quenchers across phospholipid bilayers, and of the quenching of fluorophores either free in solution, or entrapped in membranous vesicles, or embedded in lipid bilayers. Two well known water-soluble quenchers have been used, iodide [6] and acrylamide [7]. The quenching of free tryptophan fluorescence, and that of an anthroyloxy fatty acid have been considered.

*Corresponding author. Fax: (34) (4) 464 8500.

*Abbreviations:* EYPC, egg-yolk phosphatidylcholine; PC, phosphatidylcholine; 12-AS, 12-(9-anthroyloxy) stearic acid; LUV, large unilamellar vesicles; MLV, multilamellar vesicles.

## 2. MATERIALS AND METHODS

Egg-yolk phosphatidylcholine (EYPC) was grade I from Lipid Products (South Nutfield, UK). Other lipids were from Sigma. Acrylamide was ultrapure quality from Boehringer Mannheim, and NaI was analytical quality from Merck. 12-(9-Anthroyloxy) stearic acid (12-AS) was supplied by Molecular Probes.

Multilamellar vesicles (MLV) were used in permeability studies. They were prepared essentially as described by Bangham et al. [8], and left to equilibrate overnight at 4°C prior to their use. Large unilamellar vesicles (LUV) were prepared by the extrusion method, using 0.1 $\mu$m Nuclepore filters. The phospholipid concentration was determined, as phosphorous, according to Bartlett [9]. In all experiments, the aqueous medium consisted of 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, to which other solutes were added when required.

Fluorescence measurements were performed on a MPF 66 Perkin-Elmer spectrofluorometer. The solution in the cuvette was continuously stirred and, unless otherwise stated, kept at 22°C. The excitation wavelength was 290 nm (3 nm slit width), and the emission was read, unless otherwise stated, at 350 nm (3 nm slit width). Fluorescence intensity readings were corrected, when applicable, for vesicle light scattering, dilution factors and inner-filter effects [5].

Fluorescence quenching was achieved by adding increasing amounts of NaI [6] or acrylamide [7] to the cuvette. NaI solutions were freshly prepared before use in order to avoid $I^{3-}$ formation. Acrylamide quenching experiments were carried out at an excitation wavelength of 295 nm instead of 290 in order to reduce acrylamide absorbance. Data were analyzed according to the Stern–Volmer equation for collisional quenching [6]:

$$F_o/F = 1 + K_{sv}\,[Q]$$

where $F_o$ and $F$ are the fluorescence intensities in the absence and presence of the quencher, Q, and $K_{sv}$ is the Stern–Volmer quenching

constant. When appropriate, static quenching, i.e. quenching resulting from the formation of a nonfluorescent ground state complex between fluorophore and quencher, was also taken into account, and the following equation used [7]:

$$F_o/F \exp(V[Q]) = 1 + K_{sv}[Q]$$

$V$ being the so-called static quenching parameter.

Liposome permeability to quenchers was studied through the swelling of multilamellar vesicles [10]. Solute entry inside the liposome leads to an increase in volume, in turn proportional to a decrease in apparent absorbance, or turbidity ($A_{450}$). Changes in $A_{450}$ with time were followed on a Uvikon 860 Kontron spectrophotometer, equipped with thermostatted, stirred cuvettes. 20 $\mu$l of MLV suspension (0.25 mM in lipid), prepared by dispersing the lipid in 50 mM choline chloride, 50 mM Tris-HCl, pH 7.0, were added to a 2 ml solution of either 100 mM acrylamide, 50 mM Tris-HCl, pH 7.0, or 50 mM NaI, 50 mM Tris-HCl, pH 7.0.

Fluorescence lifetime measurements were carried out with an SLM-Aminco 48000 phase modulation spectrofluorometer at room temperature.

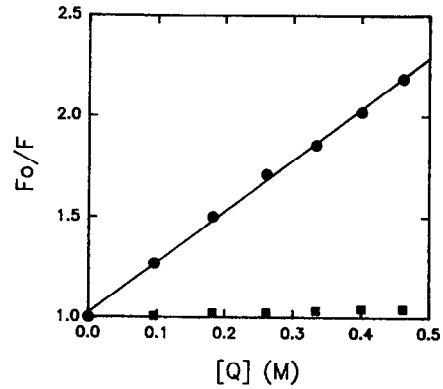


Fig. 1. Stern–Volmer plots of aqueous quenching by iodide (●) or acrylamide (■) of 12-AS incorporated into phospholipid bilayers. LUV were formed containing EYPC and 12-AS (100:1 mol ratio). 12-AS concentration in the cuvette was 7.5 $\mu$M. Iodide data were subjected to a linear fit according to the Stern–Volmer equation. A 2.5 $M^{-1}$ value for $K_{sv}$ was obtained.

## 3. RESULTS AND DISCUSSION

As a first approximation, we intended to study the accessibility to polar quenchers of a fluorophore deeply embedded in the hydrophobic matrix of the lipid bilayer. 12-(9- Anthroyloxy) stearic acid (12-AS) is known to be one such fluorophore [11–13]. (To the authors' knowledge, there is not a simple system with a single tryptophanyl residue whose hydrophobic location is known with certainty as for 12-AS). The results in Fig. 1 clearly demonstrate that 12-AS fluorescence is collisionally quenched by iodide, but not affected at all by acrylamide (see figure legend for experimental details).

The observed behaviour cannot be explained as a difference in bilayer permeability to $I^-$ or acrylamide. The swelling experiment in Fig. 2A shows that both quenchers may pass across the membrane; the non-permeant choline chloride is shown as a control. Measurements over a range of temperatures and with different lipid compositions (Fig. 2B) confirm that, under a variety of circumstances, the diffusion rates of $I^-$ and acrylamide are very similar to each other. From the experiments as a function of temperature, the corresponding Arrhenius plots have been constructed, whose slopes, representing the energies of activation for the diffusion process, are collected in Table I. $E_a$ values are similar for both quenchers when the bilayer consists of EYPC ± cholesterol, but they are smaller for acrylamide than for $I^-$ when saturated lipids are used. From these and the above results, it is concluded that, although both iodide and acrylamide can pass readily the membrane bilayer, only iodide acts as a quencher in the hydrophobic matrix, acrylamide thus being unable to quench 12-AS fluorescence in the experiment shown in Fig. 1. Consequently, fluorophores embedded in the bilayer hydrophobic matrix appear to be insensitive to acrylamide but potentially sensitive to iodide.

Subsequent observations were carried out on pure tryptophan in aqueous solution, either free or encapsulated in LUV. Vesicles prepared in 15 mM Trp were freed from non-entrapped fluorophore by passing them through a Sephadex G-75 column; independent measurements indicated that, under the conditions of our experiment, Trp leakage was negligible. The data in Fig. 3 show, as expected, that both $I^-$ and acrylamide can act on the entrapped Trp. However, the corresponding $K_{sv}$ (Table II) are larger for free than for entrapped Trp, suggesting that the fluorophore accessibility to the quencher is reduced inside the vesicle. Changes in $K_{SV}$ are not due to different fluorescence lifetimes; measurements of lifetimes of the major component of Trp fluorescence in either free or liposomally entrapped form gave respectively the values of 2.9 and 3.2 ns. Again, we observe that the bilayer does not constitute a preferential barrier for any of the quenchers. Also to be noted from Tables I and II is the negligible influence of cholesterol on the passive diffusion of iodide or acrylamide.

The observation that acrylamide is unable to quench deeply buried fluorophores (Fig. 1) confirms previous studies [13]; in addition, acrylamide has been described as unable to penetrate the lipid bilayer [14]. However, direct permeability measurements (Fig. 2) show that both acrylamide and iodide have similar permeabilities across pure lipid membranes. Studies as a function of temperature allow the calculation, through Arrhenius plots, of the corresponding energies of activation (Table I), that can be compared to those of well-known permeants, e.g. polyalcohols. De Gier et al. [10] computed 4.64 kJ/mol for glycerol, and 4.98 kJ/mol for erythritol penetration of a bilayer with a liquid paraffin core. Moreover, studies of the fluorescence quenching of Trp encapsulated in PC liposomes (Fig. 3 and Table II) demonstrate unequivocally that permeation through bilayers is not the obstacle preventing acrylamide quench-

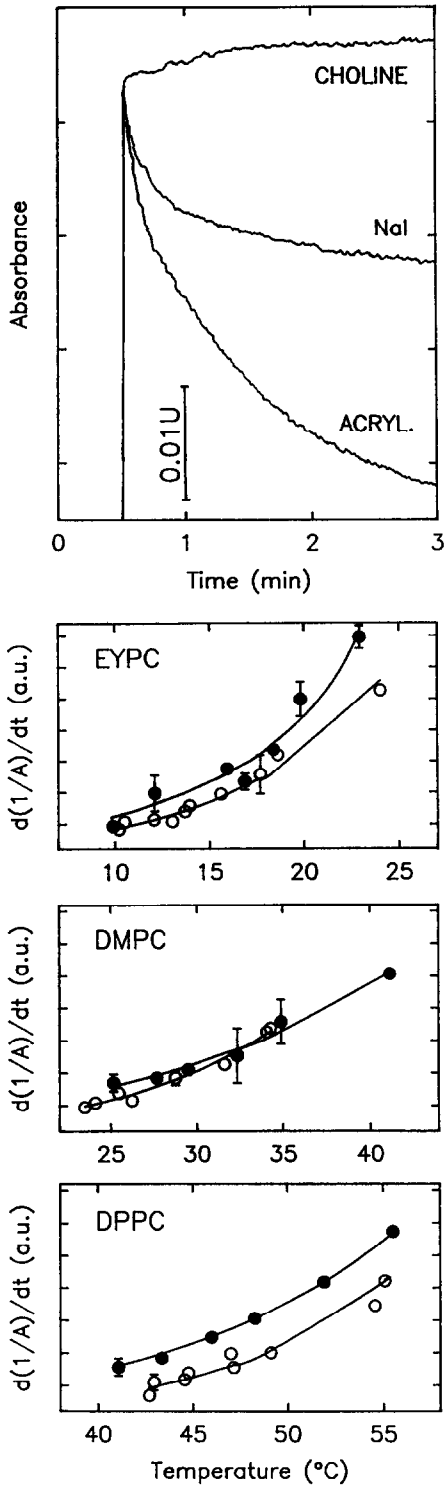


Fig. 2. Swelling of multilamellar vesicles in the presence of iodide or acrylamide. (A) Representative traces of the decrease in $A_{450}$, indicating liposomal swelling, when MLV are diluted in a medium containing 50 mM sodium iodide, or 100 mM acrylamide, or 50 mM of the non-permeant solute choline chloride, all of them in 50 mM Tris-HCl, pH 7.0. The molar concentration of acrylamide was made twice that of NaI or choline chloride in order to obtain roughly similar osmotic pressures in all three cases. The vesicles contained 50 mM choline chloride in 50 mM Tris-HCl, pH 7.0. (B) Initial swelling rates of MLV in the presence of iodide (○) or acrylamide (●). Initial swelling rates are measured from traces as shown in Fig. 2A, as $d(1/A)/dt$ [10]. MLV composition: EYPC (top), DMPC (middle), DPPC (bottom).

Table I

Energies of activation ($E_a$) for the passive diffusion of iodide and acrylamide

| Bilayer composition | $E_a$ (kJ/mol) | |
|---|---|---|
| | Iodide | Acrylamide |
| EYPC | 5.36 | 5.21 |
| Dipalmitoyl PC[a] | 5.98 | 3.66 |
| Dimyristoyl PC[a] | 4.85 | 2.66 |
| PC/cholesterol (2:1) | 5.69 | 5.17 |

[a]Values shown in the table are for temperatures above the gel-to-fluid transition.

ing of fluorophores in the lipid matrix. It can thus be concluded that, for fluorophores embedded in the hydrophobic membrane core, fluorescence quenching depends on factors other than the mere ability of the quencher to permeate across the membrane, i.e. factors that determine, ultimately, the nature of the interactions between quencher and membrane components in the liquid-crystalline state.

A final word of caution should be said on the applicability of the present observations to complex membrane protein systems, in which seemingly contradictory data may be found. To mention but one example, we have recently shown, in the mitochondrial uncoupling protein [15], fluorophores in relatively non-polar environments that are quenched by acrylamide and not by iodide. It is obvious that, in the very heterogeneous microenviroment of a protein fluorophore, steric, electro-

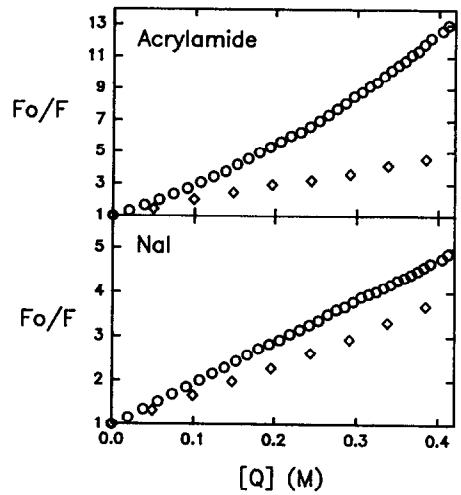


Fig. 3. Quenching of tryptophane fluorescence: Pure Trp, free in solution or entrapped in EYPC liposomes. LUV were prepared after hydrating the lipid in 15 mM Trp, 50 mM Tris-HCl, pH 7.0; external Trp was removed by elution through a Sephadex G-75 column. LUV concentration in the spectroscopic cuvette was equivalent to 0.1 mM EYPC. To 2 ml of the liposome suspension 10 μl aliquots of quencher solution were added. Top, acrylamide; bottom, iodide. (○) Trp in aqueous solution; (◇) Trp in liposomes. The data can be adjusted to Stern–Volmer equations; the corresponding parameters are shown in Table II.

Table II

Stern–Volmer quenching constants ($K_{sv}$) and static quenching parameters ($V$) for free and LUV-entrapped Trp

| Quencher | System | $K_{sv}$ ($M^{-1}$) | $V$ ($M^{-1}$) |
|---|---|---|---|
| Acrylamide | Trp in buffer | 15.8 | 1.4 |
| Acrylamide | Trp in buffer + empty liposomes | 16.2 | 1.5 |
| Acrylamide | Trp in EYPC liposomes | 9.1 | – |
| Acrylamide | Trp in EYPC/cholesterol (2:1) liposomes | 8.9 | – |
| Iodide | Trp in buffer | 9.4 | – |
| Iodide | Trp in buffer + empty liposomes | 9.6 | – |
| Iodide | Trp in EYPC liposomes | 7.4 | – |
| Iodide | Trp in EYPC/cholesterol (2:1) liposomes | 6.9 | – |

Experimental conditions as in Fig. 3.

static and other factors, that are almost irrelevant in the present study, may play a significant role in fluorescence quenching.

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