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FLUORESCENCE QUENCHING IN MEMBRANE PHASE

Zsuzsa Lakos[1], Ágnes Szarka and Béla Somogyi

Department of Biophysics, University Medical School of Pécs
H-7624 Pécs, Szigeti 12., Hungary

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Membrane-related events can be investigated when the fluorescence of an intramembrane fluorophore is quenched by molecules that are dissolved in lipid phase. In this case the bimolecular quenching constant characterises the relative transport rate of the fluorophore and quencher molecules in the membrane interior and thereby it is related to the dynamics or structure of the membrane. Unlike classic quenching experiments, the crucial point in such studies is that the concentration of the quencher in the lipid phase differs from that in the bulk. As a consequence, it is usually described by different models, or regarded as the total concentration added. Here a simple fluorometric study is presented for distinguishing between the solvation mechanisms (partition or binding) of quencher molecules in membrane phase.

Two models are known for describing the concentration of externally added quenchers inside the membrane: according to the first model the quencher molecules partition between the membrane and aqueous phase [1] whereas the second model assumes binding sites for the quencher molecules within the membrane [2].

Since the appearance of these models a number of membrane quenching studies has been evolved: some authors treat the results according to the partition model [3,4], while others operate with the binding model [2,5,6], but a kind of "mixed" description occurs also frequently [7,8]. There are examples when the authors are not interested in obtaining the value of the quenching constant, thereby they do not need to deal with the quencher concentration in lipid phase [9-10].

It is important to keep in mind that whenever the quenching constants are to be determined (either in absolute values or their relative changes) the interpretation of

[1]Corresponding author. Fax No.: 36 72 314 017.

Abbreviations:
FCS, fetal calf serum; PBS, phosphate buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-trimethylammonium]6-phenyl-1,3,5-hexatriene; 12-AS, 12-(9-anthroiloxy)-stearic acid; DMSO, dimethyl-sulphoxide.

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the experimental data is model-dependent. The careful investigation of the chosen model seems to be important to arrive at the proper interpretation.

Materials and Methods

Cells

In vitro cultured continuous mouse lymphoid cell line (Sp-2/0-Ag14) was used (Flow-Labs. Ltd., UK). The cells were cultured in RPMI-1640 culture medium (Flow-Labs. Ltd., UK) containing 10% FCS (Hungarocalf, Mezőhegyes, Hungary) in 5% CO_2 humidified atmosphere at 37 ^{o}C (tissue culture incubator, Forma Scientific, USA). The cells, growing in log phase (viability >98% as proved by Tripane blue exclusion) were washed with PBS (pH = 7.40) and labelled as described.

Fluorescence quenching experiments

The cells were labelled using DPH, its cationic derivative, TMA-DPH, and 12-AS according to [11]. Naringenin (4,5,7-trihydroxyflavanone) dissolved in DMSO in different stock solutions (2 mM to 200 mM) was used as quencher. All chemicals were of analytical grade from Sigma (USA) and solvents of spectroscopic grade from Fluka (Germany), respectively. 2 µl of DMSO or each stock solution of quencher was added to 2 ml of cell suspension containing $2x10^6$ cells/ml in order to minimize solvent perturbation of cell membrane by DMSO. Intensity data were recorded when the signal has been stabilized (see results).

Steady-state fluorometry

Fluorescence intensity measurements were carried out on a Hitachi-Perkin Elmer MPF 4 fluorimeter equipped with a thermostated cuvette holder at 25 ^{o}C. Excitation wavelengths were 360 nm (DPH, TMA-DPH) or 383 nm (12-AS) and emission wavelengths were 425 nm (DPH, TMA-DPH) or 440 nm (12-AS) with 5 nm excitation and emission slits, respectively. Intensity data were collected by an IBM PC/XT connected to the fluorometer through an AD converter.

Time-resolved fluorometry

Fluorescence lifetime measurements were performed on ISS K2 multifrequency phase fluorimeter (ISS Fluorescence Instrumentation, Champaign, Illinois, USA) using the frequency cross-correlation method. A 300 W xenon arc lamp was used as an excitation light source. The excitation light intensity was modulated using a double-crystal Pockels cell and a two-way polarizer. 80 Hz was used as a cross-correlation frequency. Excitation wavelength was set to 360 nm. The emission was monitored through a 400 nm high-pass filter (Jena G241g). Lifetime measurements were performed with the ISSL Decay Acquisition Software. Freshly prepared glycogen (Sigma, USA) solution was used as a reference to correct for the instrumental phase delay of the detection equipment. 10 phase and modulation data were collected at each selected frequency. Analysis of phase and modulation data was carried out by the ISS187 Decay Analysis Software.

Theory

In this section we are comparing two particular models (partition and binding) in order to find a way to differentiate between them by the use of experimental data set and avoid misinterpretations.

Partition model

The partition model assumes an infinite number of zero affinity binding sites for the quencher molecules within the membrane. The quencher concentration in lipid

phase varies linearly with the total concentration added into the aqueous phase. The quenching theory yields the following equation for quenchers partitioning into the bilayer [1,12-14]:

$$k_{app}=\frac{1}{\alpha_L+\frac{1}{Pk_q}}=k_q\frac{P}{\alpha_L P+1} \quad (1)$$

where k_{app} is apparent bimolecular quenching constant, α_L is the volume fraction of the membrane, k_q is the bimolecular quenching constant of the quencher in the membrane and P is the partition coefficient of the quencher between the membranous and aqueous phase. P can be defined as

$$P=\frac{[Q]_{membrane}}{[Q]_{water}}=\frac{Q_L}{Q_W}>>1 \quad (2)$$

where Q_L and Q_W refer to the concentration of the quencher in the lipid and aqueous phase, respectively. The Stern-Volmer equation can be written using the appropriate quenching constants in the following way:

$$\frac{F_0}{F}=1+k_q\tau_0Q_L=k_{app}\tau_0Q_T \quad (3)$$

where k_q indicates the bimolecular quenching constant, τ_0 is the lifetime of the excited state of the fluorophore in the absence of the quencher, kapp is the apparent quenching constant, F_0 and F are the fluorescence intensities measured in the absence and presence of quencher molecules.

The total amount of quencher molecules can be given as the sum of the amount of the quencher in the lipid and aqueous phase, respectively:

$$V_TQ_T=V_WQ_W+V_LQ_L \quad (4)$$

where Q_T reflects to the total concentration of quencher molecules and V_T, V_W and V_L are the corresponding volumes (total, water and lipid phase volume). If we define the volume fractions $\alpha_W=V_W/V_T$ and $\alpha_L=V_L/V_T$, the total concentration of the quencher can be expressed as

$$Q_T=\alpha_wQ_w+\alpha_LQ_L \quad (5)$$

Since the partition coefficient P equals to Q_L/Q_W, the form of the above equation using P is:

$$Q_T=\alpha_W\frac{Q_L}{P}+\alpha_LQ_L=Q_L\left(\frac{\alpha_W}{P}+\alpha_L\right) \quad (6)$$

Combining eqs. (3) and (6) we arrive at:

$$\frac{F_0}{F}=1+k_q\tau_0Q_L=1+k_q\tau_0\frac{Q_T}{\frac{\alpha_w}{P}+\alpha_L} \quad (7)$$

After arranging the above equation we get:

$$\left(\frac{F_0}{F}-1\right)^{-1}=\frac{1}{k_q\tau_0Q_T}\left(\frac{\alpha_W}{P}+\alpha_L\right) \quad (8)$$

As $\alpha_W \sim 1$ ($\alpha_W = V_W/V_T$, and $V_W \approx V_T$), eq. (8) can be written as:

$$\left(\frac{F_0}{F}-1\right)^{-1} = \frac{1}{k_q \tau_0 Q_T}\left(\alpha_L + \frac{1}{P}\right) \tag{9}$$

As a result, the plot of $(F_0/F\text{-}1)^{-1}$ as a function of $1/Q_T$ results in a linear relationship with zero intercept.

Binding model

This model assumes binding sites of definite number and affinity for the quencher molecules in the interior of the membrane. The quencher concentration in lipid phase follows a saturation profile with the total concentration added. In this case the following binding equilibrium can be written [2]:

$$Q + S \rightleftharpoons S_b \tag{10}$$

$$K_D = \frac{[Q][S]}{[S_b]} \tag{11}$$

where Q is the concentration of free quencher in aqueous phase, S is the concentration of free binding sites within the membrane, S_b is the concentration of binding sites occupied by quencher molecules and K_D is the dissociation constant. The total amount of binding sites in the membrane, S_T, can be given as:

$$S_T = S_{bound} + S_{free} \tag{12}$$

Combining eqs. 11. and 12. we get the following expression for S_b:

$$[S_b] = \frac{[Q][S_T]}{K_D + [Q]} \tag{13}$$

If we assume that $Q_T >> S_T$ (in excess of quencher) the value of Q can be replaced by Q_T, so eq. (13) takes the form of

$$[S_b] = \frac{[Q_T][S_T]}{K_D + Q_T} \tag{14}$$

Since the concentration of the free quencher molecules in the membrane is negligible (otherwise there would be partition instead of binding), the appropriate form of the Stern-Volmer equation for this case is:

$$\frac{F_0}{F} = 1 + k_q \tau_0 S_b = 1 + k_q \tau_0 \frac{Q_T S_T}{K_D + Q_T} \tag{15}$$

Eq. (14) can be rearranged to obtain a form comparable with eq. (9) as

$$\left(\frac{F_0}{F}-1\right)^{-1} = \frac{1}{k_q \tau_0}\left(\frac{K_D}{Q_T S_T} + \frac{1}{S_T}\right) = \frac{1}{Q_T}\frac{K_D}{k_q \tau_0 S_T} + \frac{1}{k_q \tau_0 S_T} \tag{16}$$

After plotting the corresponding $(F_0/F\text{-}1)^{-1}$ values as a function of $1/Q_T$, we obtain a linear relationship with the intercept of $1/k_q \tau_0 S_T > 0$.

Results

The fluorescence intensity of the fluorophore-labelled cells was decreased upon addition of quencher for 15-20 min until it reached a steady value reflecting the time necessary for the penetration of quencher molecules into the bilayer (data not shown).

Figure 1. shows the results of steady-state measurements. The experimental data plotted according to eqs. (9) and (16) are on a linear curve with a non-zero intercept indicating that our experimental system can be described by the binding model. From the parameter of the linear relationship the binding constant can be determined according to eq. (16). (see table 1.)

Figure 2. shows time-resolved data DPH as a fluorophore. It also supports the existence of the binding mechanism. The value of the binding constant calculated from the parameters of the fitted line according to eq. (16) is 10.6 μM which is comparable to the average value of 20.6 μM obtained by steady-state measurements. The fact that the $(F_0/F\text{-}1)^{-1}$ and $(\tau_0/\tau\text{-}1)^{-1}$ values fall in the same range at both type of measurements indicates that the quenching is predominantly of collisional type.

The above results show that in our experimental system the presence of quencher molecules in lipid phase is preferentially due to binding. The theory section reveals that the plot of $(F_0/F\text{-}1)^{-1}$ versus $1/Q_T$ gives a linear relationship for both models (partition and binding). The special characteristics of the two particular descriptions permit to distinguish between them. The inspection of eqs. (9) and (16)

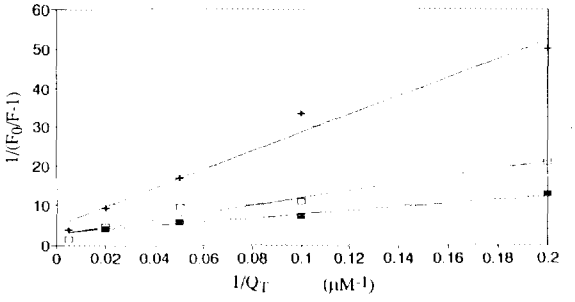

Figure 1.
The plot of the quenching parameter $(F_0/F\text{-}1)^{-1}$ as a function of the reciprocal of the total quencher concentration, $1/Q_T$, determined using DPH, TMA-DPH and 12-AS as fluorescent lipid probes embedded in the membrane of Sp-2/0-Ag14 cells and naringenin as quencher at 25 °C. The curves confirmed that the association mechanism is binding in this experimental system. From the slope and intercept of the fitted lines the dissociation constant of quencher molecules, K_D, can be determined (see eq. (16)). Note that the number of cells per ml (i.e., the total number of binding sites, S_b) is kept constant during the measurements (see Materials and Methods section).
TMA-DPH (■ - ■); DPH (□ - □); 12-AS (✦ - ✦).

Table 1

	DPH	TMA-DPH	12-AS
	19.0	19.9	13.1
K_D	10.4	17.6	19.6
(μM)	19.8	28.8	24.8
	23.7		
	30.1		
average ± SD		20.6 ± 5.7	

Values of the dissociation constant, K_D, obtained from a series of steady-state quenching experiments according to eq. (16). The fluorescence of the lipid probes DPH, TMA-DPH and 12-AS embedded in membrane of Sp-2/0-Ag14 cells was quenched by applying naringenin in different concentrations.

clearly indicates that this kind of differentation is absolutely necessary in order to avoid misinterpretation. Eqs. (9) and (16) also show that the relative change of k_q can be determined without knowing the parameters S_T, or α_L and P. For such a relative comparison, however, the appropriate parameter of the above plots (the slope for partition and the intercept for binding) should be used.

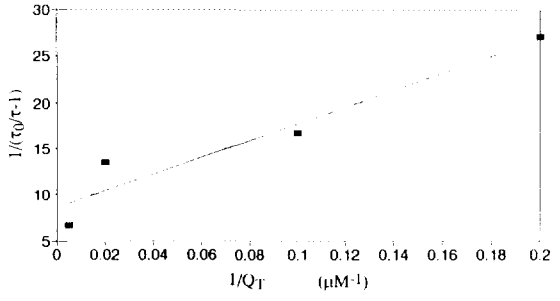

Figure 2.
The plot of the quenching parameter $(\tau_0/\tau\text{-}1)^{-1}$ as a function of the reciprocal of the total quencher concentration, $1/Q_T$, determined using DPH as a fluorescent lipid probe embedded in the membrane of Sp-2/0-Ag14 cells and naringenin as quencher at 25 °C. The resulting curve is characteristic for the binding mechanism as the intercept differs from zero. From the slope and intercept of the fitted lines the dissociation constant of quencher molecules, K_D, can be determined (see eq. (16)).

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References

1 Lakowicz, J.R., and Hogen, D. (1980) Chem. Phys. Lipids, 26, 1-40.
2 Verkman, A.S. (1980) Biochim. Biophys. Acta, 599, 370-379.
3 Chalpin, D. B., and Kleinfeld, A. M. (1983) Biochim. Biophys. Acta, 731, 465-474.
4 Kühn-Velten, W. N., and Kempfle, M. A. (1993) Biochim. Biophys. Acta, 1145, 185-190.
5 Cabrini, G., and Verkman, A. S. (1986) Biochim. Biophys. Acta, 862, 285-293.
6 Louro, S. R. W., Nascimento, O. R., and Tabak, M. (1994) Biochim. Biophys. Acta, 1190, 325-328.
7 Haigh, E. A., Thulborn, K. R., and Sawyer, W. H. (1979) Biochemistry, 18, 3525-3532.
8 Vermeir, M., and Boens, N. (1992) Biochim. Biophys. Acta, 1104, 63-72.
9 Cranney, M., Cundall, R. B., Jones, G. R., Richards, J. T., and Thomas, E. W. (1983) Biochim. Biophys. Acta, 735, 418-425.
10 Van Amersfoort, E. S., and Van Strijp, J. A. G. (1994) Cytometry, 17, 294-301.
11 Lakos, Zs., Somogyi, B., Balázs., M., Matkó, J., and Damjanovich, S. (1990) Biochim. Biophys. Acta, 1023, 41-46.
12 Lakowicz, J.R., and Hogen, D. (1977) In Membrane Toxicity (M. W. Miller and A. E. Shamoo, Ed.s), pp. 509-546. Plenum Publishing Corp., New York.
13 Lakowicz, J.R., Hogen, D., and Omann, G. (1977) Biochim. Biophys. Acta, 471, 401-411.
14 Omann, G. M., and Glaser, M. (1985) Biophys. J., 47, 632-627.