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Vertical fluctuations of phospholipid acyl chains in bilayers

John R. Wardlaw, William H. Sawyer and Kenneth P. Ghiggino

Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia

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The possibility of vertical displacement of acyl chains in lipid bilayers has been examined by quenching the fluorescence of 2-(9-anthroyloxy)palmitic acid with 5- and 16-doxylstearates in dipalmitoylphosphatidylcholine unilamellar vesicles. Measurement of lifetime and steady-state quenching showed that the dynamic component of quenching was independent of the transverse position of the quencher indicating that a quencher at the 16-position could interact with a fluorophore at the 2-position with high frequency. The differences in steady-state quenching could be accounted for by the differences in the static component of quenching. The results provide further evidence for rapid vertical displacements of acyl chains in phospholipid bilayers.

Bilayer dynamics; Fluorescence quenching; Anthroyloxy fatty acid

1. INTRODUCTION

Phospholipid molecules in lipid bilayers undergo dynamical motions of various types. Translational diffusion coefficients are of the order of 10^{-7} cm$^2 \cdot$s^{-1} [1]. Global rotation takes place about the long axis of the molecule with a correlation time within the range 10^{-9}–10^{-6} s [2], and *trans-gauche* isomerizations occur about carbon–carbon bonds along the acyl chain with a frequency of about 10^{10} s^{-1} [3]. Less well documented are bending motions and vertical fluctuations of the acyl chains which in extreme cases may result in the terminal methyl group approaching the surface of the bilayer. Evidence favouring this last possibility has come from measurements of electron-electron double-resonance interactions between ^{14}N- and ^{15}N-spin-labeled stearic acid probes in lipid bilayers. Vertical fluctuations of the acyl chains were determined by placing the labels at different positions along the acyl chain of separate fatty acid probes. It was found that a probe attached near the terminal methyl group could collide with low frequency with a probe attached near the head group region [4,5]. A more recent study using the same technique found a higher frequency of interaction [6].

Fluorescent and spin-labeled fatty acids have been used extensively to study the physical nature of microenvironments at a graded series of depths in the lipid bilayer [7,8]. Here, we examine the possibility of gross bending distortions of the acyl chains by measuring the quenching of *n*-(9-anthroyloxy) fatty acids by *n*-doxylstearates in situations where the fluorophore and quenching groups are at different transverse positions in the bilayer. Quenching is measured by both steady-state and time-resolved methods to identify the contributions of static and dynamic mechanisms to the overall quenching.

2. MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC, >99%) was obtained from Sigma. The 2- and 16-(9-anthroyloxy)palmitic acids (2-AP

Correspondence address: W.H. Sawyer, Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia

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and 16-AP) were synthesized from anthracene 9-carboxylic acid and the appropriate *n*-(hydroxy) fatty acid by anhydride synthesis [9], and were purified by preparative thin-layer chromatography [10]. *n*-Doxylstearates (*n*-NS) were obtained from Molecular Probes (OR). The relative concentrations of the spin probes were determined by double integration of the first-derivative ESR spectrum.

Unilamellar phospholipid vesicles were prepared according to established procedures [10]. Vesicles were cycled back and forth across their mesomorphic phase transition three times to remove defects in the bilayer structure [11]. *n*-Anthroyloxy fatty acids were incorporated into vesicles by adding a small volume (10 μl) of a stock solution (1 mM) in methanol to a suspension of vesicles (2.5 ml, 0.5 mM DPPC). Uptake was for 1 h at 35°C in the dark. *n*-NS quenchers were added as 5-μl aliquots of a methanolic stock solution (1.6–2.6 mM).

Steady-state fluorescence measurements were made with a Perkin Elmer MPF3 or LS5 spectrofluorometer. To compare the quenching efficiency of the *n*-NS probes, Stern-Volmer plots were made in which the concentration of quencher in the bilayer was represented on the abscissa axis. This concentration depends on the partition coefficient of the quencher between the aqueous and lipid compartments. The partition coefficients were determined by measuring the dependence of the quenching efficiency on the volume fraction of the lipid phase, as described [3].

Fluorescence lifetimes were measured on an Ortec/Applied Photophysics SP2X nanosecond spectrometer using the technique of time-correlated single-photon counting. The excitation wavelength was 365 nm and the emission was isolated by a Gena-GG 400 nm cut-off filter. Details of the apparatus and data analysis procedures have been described in [13]. Fluorescence lifetimes were obtained from a single-exponential fit of the decay profile.

For dynamic quenching, the change in fluorescence is related to the quencher concentration by the Stern-Volmer relationship:

$$\frac{I_0}{I} - 1 = \frac{\tau_0}{\tau} - 1 = K_{SV}[Q] = k_q\tau_0[Q] \quad (1)$$

where I and I_0 are the fluorescence intensities in the presence and absence of quencher, τ and τ_0 are the corresponding fluorescence lifetimes, K_{SV} is the Stern-Volmer quenching constant, and k_q is the bimolecular rate constant. When both static and dynamic processes compete effectively for the quenching process, the quenching is described quantitatively by [14]:

$$\frac{I_0}{Ie^{V[Q]}} - 1 = K_{SV}[Q] \quad (2)$$

where V is the static quenching constant. For the active sphere model of static quenching, V is related to the radius (R) of the sphere by the approximation, $R = (400V)^{1/3}$ [15]. Combining eqns 1 and 2 provides:

$$\ln\frac{I_0}{I} - \ln\frac{\tau_0}{\tau} = V[Q] \quad (3)$$

V was determined by plotting the left-hand side of eqn 3 vs [Q]: k_q was determined directly from the lifetime data (eqn 1).

3. RESULTS

The steady-state fluorescence quenching of 2-AP by 5-NS and 16-NS in DPPC vesicles is depicted in fig.1. The small but finite amount of quenching observed for the 2-AP/16-NS fluorophore/quencher pair is perhaps surprising, since for an extended configuration of the acyl chain the anthroyloxy group would be centred about 12.4 Å away from the 16-positioned doxyl group on the quencher. Quenching of the fluorophore by 5-NS and 16-NS would require lateral diffusion of both quencher and fluorophore, but in addition, dynamic interaction between 2-AP with 16-NS would require bending of the acyl chain to bring the 16-positioned doxyl group close to the 5-positioned fluorophore. If such bending motions occur with low frequency compared to interactions controlled by the lateral diffusion, we might expect that the differences in quenching efficiency would be reflected in the values of the bimolecular rate constant. Lifetime quenching studies were therefore carried out to determine the bimolecular rate constant for the dynamic interaction of each fluorophore/quencher pair. The experiments were conducted above

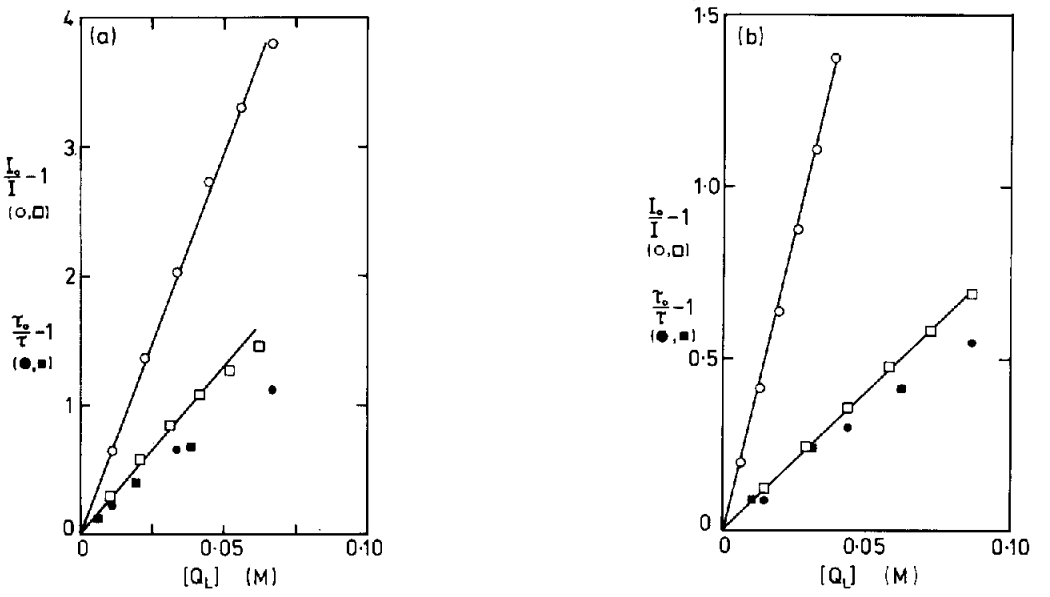

Fig.1. The quenching of 2-(9-anthroyloxy)palmitic acid by 5- and 16-doxylstearates in DPPC vesicles at (a) 28°C and (b) 48°C. (○, □) Steady-state quenching, (●, ■) lifetime quenching. (○,●) 5-NS, (□, ■) 16-NS. [Q_L], concentration of quencher in the lipid phase. Phospholipid concentration, 300 μM.

(48°C) and below (28°C) the main phase transition of DPPC vesicles (41°C).

The results are summarized in table 1 and we draw attention to the following features:

(i) The partition coefficients of 5-NS and 16-NS are greater at 48°C than at 28°C. At each temperature, 16-NS has a much lower partition coefficient than 5-NS.

(ii) The values of K_{SV} reflect the steady-state quenching efficiencies for the two quenchers (i.e. 5-NS > 16-NS). However, values of K_{SV} are lower at 48°C than at 28°C presumably due to higher nonradiative deactivation at the higher temperature as reflected in the lower lifetime of the fluorophore.

(iii) At both temperatures the value of k_q is independent of the transverse position of the fluorophore. This is also apparent in fig.1 which shows little difference in the lifetime quenching by 5-NS and 16-NS at both temperatures.

Table 1

Quenching of 2-anthroyloxypalmitic acid in dipalmitoylphosphatidylcholine bilayer vesicles by *n*-nitroxystearates (*n*-NS)

Temperature (°C)	Quencher	K_p[a]	K_{sy} (M^{-1})	τ_0[b] (ns)	K_q (10^9 $M^{-1}\cdot s^{-1}$)	V
28	5-NS	12570	57.8	8.3	2.0	11.3
	16-NS	3340	37.7		2.1	9.2
48	5-NS	89000	15.8	5.7	1.1	4.8
	16-NS	9730	11.1		1.2	2.9

[a] Partition coefficient determined by the method of Blatt and Sawyer [7]
[b] Fluorescence lifetime in the absence of quencher

(iv) At both temperatures the value of V is significantly less for 16-NS than for 5-NS.

Additional experiments involved the quenching of 12-(9-anthroyloxy)stearic acid (12-AS) and 16-AP with 5-NS and 16-NS. In all cases, static quenching was the major contributor to the difference in the steady-state quenching efficiency which reflected the transverse positions of fluorophores and quenchers. The dynamic component remaining relatively constant.

4. DISCUSSION

The most important feature of the results is that the difference in quenching efficiency between 5-NS and 16-NS can be accounted for almost entirely by the differences in the static component of quenching. The dynamic quenching appears to be independent of the transverse position of the quenching group. This is true irrespective of the phase state of the lipid. The result implies that the frequency of the interaction of each quencher with 2-AP is similar, and that the deeper transverse position of the 16-doxyl group does not inhibit its interaction with the fluorophore closer to the bilayer surface. Indeed, chain bending, which would be required to accomplish this interaction, must occur with a frequency comparable to the frequency of interaction due to lateral diffusion in the bilayer. The association-dissociation of probe molecules between aqueous and lipid compartments may also contribute to fluorophore-quencher interactions as probe molecules enter and leave the bilayer.

In the double resonance study referred to in section 1 [6], the frequency of interaction between C5 and C16 probes was about half the frequency of C16:C16 collisions. The failure in the present study to find any difference between the bimolecular rate constants for C2:C5 collisions and C2:C16 collisions is therefore unusual. Part of this difference may be due to the bulkiness of the anthracene fluorophore relative to the doxyl group so that less of an excursion of the acyl chain is required to bring the doxyl group close to the anthracene ring. An additional factor concerns the use of Stern-Volmer theory to describe quenching in an anisotropic lipid bilayer. It is now known that this is not always appropriate [16], although the deviations from Stern-Volmer behaviour would be minimal for the anthroyloxy fatty acid probes which have relatively low lifetimes (7–12 ns) and slow translational diffusion ($< 10^{-6}$ cm$^2 \cdot$ s^{-1}).

The differences in steady-state quenching observed at each temperature are due almost entirely to the static component of quenching. For the 'active sphere' model of static quenching [15], instantaneous quenching is assumed to occur if the quencher molecule approaches to within a given radius of the fluorophore. The radius of this sphere is characteristic for a given fluorophore/quencher pair. However, this model assumes that the distributions of quencher and fluorophore are homogeneous as would occur in free solution. This is not the case in the lipid bilayer where both quencher and fluorophore are compartmentalized within the lipid phase. Thus, the differences observed in the apparent static quenching constant (V) are a consequence of this compartmentalization and reflect differences in the local concentration of quencher molecules across the transverse plane of the bilayer rather than differences in the radius of the sphere of influence.

In summary, the fluorescence quenching results support the view that significant vertical displacements of the acyl chains occur in phospholipid bilayers. These motions appear to take place on the fluorescence time scale (10^{-8}–10^{-9} s). It will be important to determine if such motions are affected by the presence of membrane protein.

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