Measurement of the lateral diffusion coefficients of ubiquinones in lipid vesicles by fluorescence quenching of 12-(9-anthroyl)stearate

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The lateral diffusion coefficients of some ubiquinone homologues have been measured in phospholipid vesicles exploiting the fluorescence quenching of the probe 12-(9-anthroyl)stearate by the quinones. Diffusion coefficients higher than 10^{-6} $cm^2 \cdot s^{-1}$ have been found at 25°C, compatible with the localization of the ubiquinones in the low-viscosity midplane region of the bilayer.

Ubiquinone *Phospholipid bilayer* *Fluorescence quenching* *Lateral diffusion*

1. INTRODUCTION

Since the original proposal by Green [1] that ubiquinone acted as a mobile electron carrier, the view that ubiquinone in mitochondrial and bacterial membranes and plastoquinone in chloroplasts act as a common pool and react in a functionally homogeneous fashion with the electron transfer complexes has been widely accepted. Kröger and Klingenberg [2] have provided strong kinetic evidence for the pool function of ubiquinone in mitochondrial membranes; moreover Schneider et al. [3], by phospholipid enrichment of mitochondrial membranes, have determined that a diffusion-mediated step exists in the electron transfer from dehydrogenases to bc_1 cytochromes.

No direct measurement of the lateral diffusion coefficients for ubiquinone is available at present. However, Hackenbrock et al. [4], using a fluorescent derivative of ubiquinone-2 and the method of fluorescence photobleaching recovery, reported values of the order of 10^{-9} cm^2/s that is a surprisingly low value, but still faster than the turnover of the mitochondrial respiratory chain and compatible with the pool function of ubiquinone as a mobile component [4].

We have approached the problem of lateral diffusion of ubiquinone with the rationale of using natural ubiquinones rather than using modified forms thereof. We have used a method involving fluorescence quenching, exploiting the fact that ubiquinones in their oxidised form quench the fluorescence of several chromophores. This paper reports the lateral diffusion coefficients of some ubiquinone homologues in phospholipid vesicles, obtained from the quenching of fluorescence of the probe 12-(9-anthroyl)stearic acid (AS).

2. MATERIALS AND METHODS

Phospholipid vesicles were prepared by sonication of mixed soybean phospholipids [5] (Asolectin, Associated Concentrates, New York) purified according to [6]. The fluorescent probe used was AS obtained from Molecular Probes, Junction City, OR. It was stored as a solution in absolute ethanol at −20°C at a concentration of 10^{-3} M.

The probe was incorporated into the lipid vesicles by addition of the ethanolic solution to an aqueous suspension of vesicles and then the mixture was incubated for 40 min at room temperature. Fluorescence studies were carried out with a

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Perkin Elmer MPF-4 spectrofluorimeter using an excitation wavelength of 366 nm.

Different ubiquinone homologues were a kind gift from Eisai Co., Tokyo; they were stored as a solution in absolute ethanol at −20°C at a concentration of 10 mM as determined spectrophotometrically at 275 nm using an average extinction coefficient of 12.5 $mM^{-1} \cdot cm^{-1}$ [7].

Ubiquinones were incorporated into lipid vesicles either by addition of different amounts of an ethanolic solution of quinones or by cosonication with phospholipids. The lipid-soluble spin label used to measure probe diffusion was a 16-(*N*-oxyl-4′,4′-dimethyloxazolidine) derivative of stearic acid (NS) obtained from Syva, Palo Alto, CA; NS was incorporated in phospholipid by addition of ethanolic solution to a suspension of lipid vesicles.

2.1. *Calculation of lateral diffusion from fluorescence quenching*

The probe AS can be incorporated into phospholipid vesicles and X-ray diffraction studies on lecithin bilayers have indicated that the anthracene ring of the probe is located in the hydrocarbon phase of the lecithin [8]. Ubiquinone quenches the fluorescence of AS by a collisional mechanism [9], the extent of quenching depending upon the lipid-water partition coefficient and the rate of diffusion in the lipid bilayer [10]. Therefore, the collisional quenching of fluorescence can be used for calculating the diffusion and partition coefficients of different ubiquinone homologues in phospholipid vesicles [10]. In a membrane where quenching occurs in the lipid phase and partition with the water phase may be significant, the Stern-Volmer relation for collisional quenching is modified, and the following relation holds [10]:

$$\frac{1}{K_{app}} = \alpha_m \left(\frac{1}{K_m} - \frac{1}{K_m P}\right) + \frac{1}{K_m P}$$

where K_{app} is the apparent (measured) bimolecular quenching constant in $M^{-1} \cdot s^{-1}$, K_m is the bimolecular quenching constant in the membrane phase, α_m is the volume fraction of the membrane phase and P is the partition coefficient (in molar units). A plot of $1/K_{app}$ *vs* α_m gives a straight line with $1/(K_m P)$ as intercept and $[(1/K_m) - (1/K_m P)]$ as slope.

From the K_m values the diffusion coefficients are calculated by the equation of Smoluchowski [10]:

$$K_m = 4\pi\gamma\sigma_{pq} N' (D_{pm} + D_{qm})$$

where γ is the quenching efficiency of the fraction of collisional encounters which are effective in quenching, σ_{pq} is the sum of molecular radii of the probe plus quencher, N' is Avogadro's number per mmol and D_{pm} and D_{qm} are the diffusion coefficients of the probe and quencher, respectively; the subscript is used to indicate the membrane phase. We have assumed that the quenching efficiency would be 100%; thus the diffusion coefficients calculated in this way is the smallest possible.

Using the Smoluchovski relation it is possible to calculate only the sum of the diffusion coefficients of the probe plus the quencher; we have therefore measured the diffusion of the probe independently using as quencher a spin-label, NS. This molecule is very similar to AS so they must have comparable diffusion coefficients. The values found for AS + NS diffusion have been divided by 2 and subtracted from the values of diffusion of AS + quinones.

3. RESULTS

Stern-Volmer plots of quenching of AS fluorescence by ubiquinones are usually linear (fig.1A) indicating the prevalent collisional nature of the quenching. Deviations from linearity are sometimes present at low membrane fractional volumes and/or high quinone concentrations; when present, such deviations are relatively small and have been corrected. A plot of $1/K_{app}$, as obtained by the primary plot as in fig.1A, *vs* α_m is shown in fig.1B for the homolog Q_3; from such plots, values for both the partition coefficient P and the true bimolecular quenching constant in the membrane can be obtained. The partition coefficient is calculated in $(mol_Q/mol_{PL})/(mol_Q/mol_{water})$; the P value for Q_1 is in good agreement with that calculated by Ragan [11] by either a kinetic approach or from solubility. The coefficients for the Q homologues having isoprenoid chains of two units or more are lower than those expected from the solubility values (fig.2), suggesting that part of the Q added to the system exists in a form which is not accessible for quenching, possibly high aggregates adhering to the lipid bilayers or nonmonomeric forms in the bilayer [12]. Under experimental con-

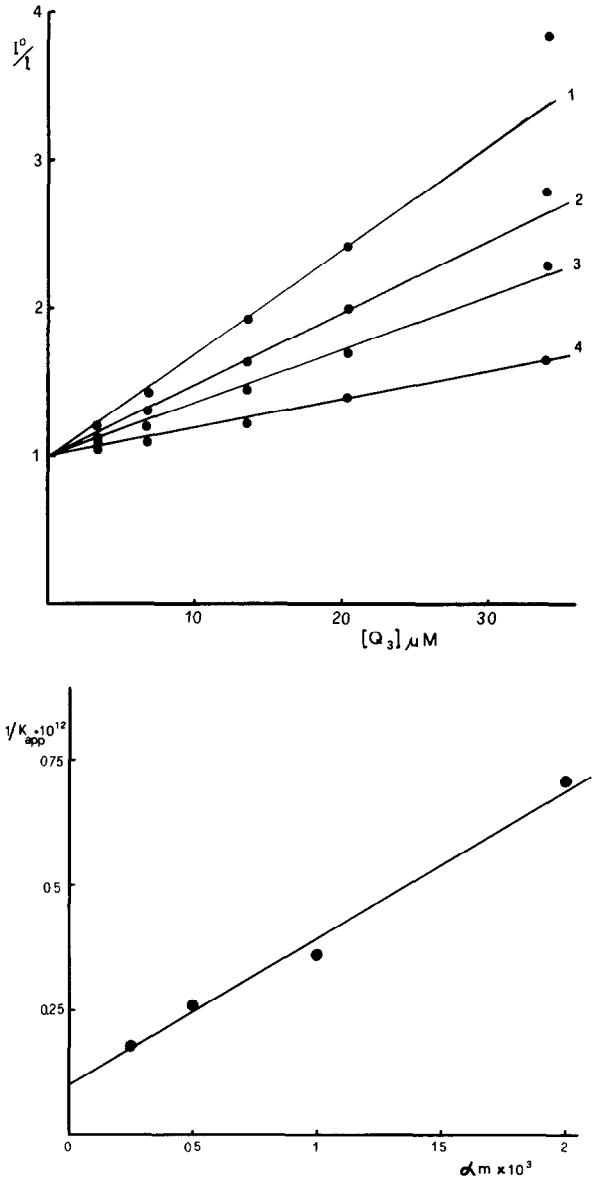

Fig.1. (A) Stern-Volmer plots of quenching of the fluorescence of 12-(9-anthroyl)stearate by Q_3 at different fractional membrane concentrations; the α_m values were as follows: curve 1, 0.25×10^{-3}; curve 2, 0.5×10^{-3}; curve 3, 1×10^{-3}; curve 4, 2×10^{-3}. (B) Plot of $1/K_{app}$ *vs* α_m using the values of panel A.

ditions for the quenching studies, using phospholipid concentrations of 0.5–4 mg/ml (as in fig.1A), the percentage of ubiquinones present in the membrane phase ranges between 85 and 98%. From the fluorescence measurements it can be noted that the

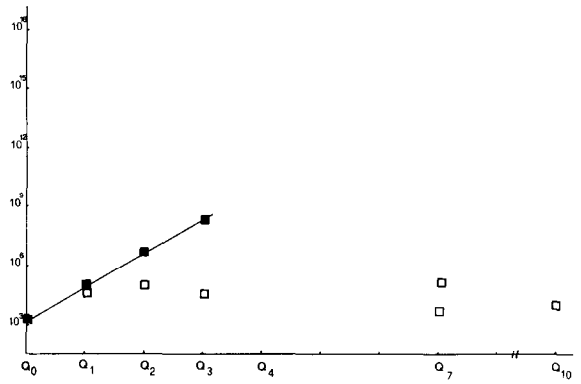

Fig.2. Partition coefficients of different Q homologues; (■) values obtained from solubility (cf. [11]); the solubility of Q_2 and Q_3 was also determined by evaluation of critical micelle concentrations (unpublished); (□) values obtained by fluorescence quenching (this study); the two values for Q_7 refer to the unsonicated and sonicated sample, respectively (cf. table 1).

manner of addition of the quinones to the lipids can modify the quenching characteristics; in particular the long-chain homologues are not able to quench AS fluorescence when added from ethanolic solutions, but only when cosonicated with the phospholipids. Since long-chain ubiquinones are incorporated into phospholipid vesicles when added from ethanolic solutions [13], this observation would suggest that a monomeric physical disposition of long-chain ubiquinones is preferred when they are cosonicated with the phospholipids.

Table 1

Partition and diffusion coefficients of some ubiquinone homologs in phospholipid vesicles at 25°C

Q homolog	Way of incorporation	$K_m \times 10^{-9}$ ($M^{-1}\cdot s^{-1}$)	$P \times 10^{-4}$ [a]	$D_{qm} \times 10^6$ ($cm^2\cdot s^{-1}$)[b]
Q_1	added[c]	3.26	9.1	4.5
Q_2	added	5.94	17.6	9.0
Q_3	added	3.64	18.5	5.2
Q_7	added	5.63	0.6	0.5
Q_7	sonication	3.87	3.2	5.5
Q_{10}	sonication	2.26	6.0	2.9

[a] (mol Q/mol phospholipid)/(mol Q/mol water)
[b] D_{qm} calculated by subtraction of D_{pm}; D_{pm} was found to be 8.5×10^{-7} by quenching of NS divided by two (see section 2)
[c] Added from concentrated ethanolic solutions

Using the Smoluchowski equation (see section 2) we have calculated from the K_m values the diffusion coefficients for the different ubiquinones. The results are reported in table 1. The lateral diffusion coefficients at 25°C of the quinones (obtained by subtracting the coefficient for the probe alone, see section 2) are very large and usually well above 10^{-6} cm^2/s. It must be stressed that in the Smoluchowski equation the use of quenching efficiency < 1 would tend to increase the diffusion values. The diffusion coefficient for the stearic acid probe is 8.5×10^{-7}, which is slightly faster than the values usually reported for the larger phospholipid molecules [14], but within the same order of magnitude.

4. DISCUSSION

The diffusion coefficient of an object moving in a membrane strongly depends upon the location of the object; for cylindrical molecules the viscosity of the fluids bathing the membrane, as well as that of the lipid bilayer, determine the diffusion rate [15–17] and equations have been devised taking account of the drag exerted by the outer fluids. For bidimensional translational motion when no viscous stress is transmitted across the surfaces there is not satisfactory solution [15]; by applying the method of irreversible thermodynamics to the Langevin equation for unsteady viscous flow [15] a diffusion coefficient can be calculated that does not take account of the viscosity of the outer medium.

The localization of ubiquinone (or plastoquinone) in the lipid bilayer of natural and model membranes has been the object of several investigations (cf. [18]); differential scanning calorimetry [12], UV spectroscopy [13], ESR [19] and NMR [20] measurements indicate that ubiquinone is located relatively deep within the hydrophobic region of the bilayer close to the midplane. This fact would suggest that the quinone lies in a region of relatively low viscosity, in accordance with the fluidity gradient detected by magnetic resonance across the membrane [21]. The midplane region could experience viscosities of less than 0.2 P and as low as 0.01 P [21]. From the rotational correlation times of a lipid spin label having the nitroxide group near the center of the bilayer, we have calculated a midplane viscosity of 0.17 P (unpublished). According to Saffmann and Delbrück [15] using their irreversible thermodynamics approach, this would correspond to a D_L for ubiquinone in the range of 5×10^{-6} cm^2/s. This is in good accordance with our experimental data.

In mitochondrial membranes, midplane viscosities are slightly higher than those in lipid bilayers, but are still compatible with D_L in the range of 10^{-6} cm^2/s [22]. The very large diffusion coefficients measured by us tend to confirm the idea that lateral diffusion of quinones is not rate-limiting for electron transfer. The lateral translational rate r/t, calculated by the relation $r^2 = 4D_L$ for a two-dimensional path and a D_L of 10^{-6} cm^2/s, would be 640 nm/ms. This indicates a diffusion rate being much faster than a rate-limiting turnover of the respiratory chain [18].

On the other hand, in the case of the chloroplast grana membranes, the diffusion properties of plastoquinone in the membrane appear to be critical for the overall rate of the electron flow [23]; the two photosystems are in fact separated over large distances in the stacked and exposed membrane areas; the mobile plastoquinone pool must connect photosystem II with the cytochrome b_6f complex, which is probably located in the exposed areas close to photosystem I [23]. It has been proposed that lateral diffusion of quinone occurs by 'tunnelling' along the midplane region and is extremely rapid with a D_L as high as 10^{-6} cm^2/s [24]. Our results offer a direct indication that such high diffusion rates are possible.

The D_L values found in mitochondria by fluorescence photobleaching recovery (FPR) of a fluorescent ubiquinone derivative are much lower [4], in the range of 10^{-9} cm^2/s, and cannot be explained solely by the presence of proteins. This discrepancy could be explained by the position of the unphysiological quinone derivative employed. This is found to be located in the glycerol region of the bilayer [4] unlike the position of the natural quinones used in this investigation. An additional explanation may lie in the fact that the FPR technique measures long-range diffusion, and not short-range diffusion, so that the presence of integral membrane proteins could increase the path length for quinone diffusion.

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