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The transverse organisation of ubiquinones in mitochondrial membranes as determined by fluorescence quenching

Evidence for a two-site model

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Abstract. The transverse organisation of ubiquinone in mitochondrial membranes was investigated by quenching a set of fluorescent fatty acids. We show that the fluorescent moiety of the probes is located at a graded series of depths in the mitochondrial membrane. The probes sense the characteristics of the lipid phase and do not significantly perturb mitochondrial function as measured by the respiratory control ratio and the ADP/O ratio. The anthroyloxy fatty acids are readily quenched by ubiquinone-10. A recently developed method in the analysis of quenching data was used to obtain the subvolume of the membrane within which the quenching interactions are confined. The results indicate that ubiquinone-10 is restricted to two sites in the transverse plane of the membrane: one near the surface and the other close to the bilayer centre. The implications of these findings for the "two-pool" model of ubiquinone organisation are discussed.

Key words: Ubiquinone, mitochondrial membrane, fluorescence quenching

Introduction

The important position of ubiquinone as a component of the respiratory chain has been amply demonstrated by depletion-repletion experiments (Ernster et al. 1969) and by the use of bacterial mutants lacking the ability to synthesize ubiquinone (Cox et al. 1970). Mitchell (1961, 1975, 1976) has proposed that ubiquinone acts as the electron and proton carrier at site II in the mitochondrial membrane and allows the vectorial transfer of protons from one side of the membrane to the other. Similar translocations have been proposed for ubiquinone in the bacterial membrane and for plastoquinone in the chloroplast membrane.The observed rate of 'flip-flop' of ubiquinone across the lipid bilayer, at least in model systems, seems to be sufficient to explain observed rates of electron transport (Futami et al. 1979).

The equilibrium distribution of ubiquinone in the transverse plane of the membrane is important, as well as its rate of movement or interchange across the bilayer between the quinone and quinol forms. Evidence from NMR experiments suggests that the quinone ring of the longer chain ubiquinones is buried deep in the bilayer, and that of ubiquinol is located closer to the bilayer surface (Kingsley and Feigenson 1981). However, the interpretation of experiments in model systems can be complicated by the fact that ubiquinone can form a separate phase in water and within the lipid bilayer itself (Quinn and Esfahani 1980; Degli Esposti et al. 1981; Katsikas and Quinn 1982). It is also possible that ubiquinone can assume more than one position or orientation within the bilayer and that the free energies associated with these positions are sufficiently similar as to ensure an equilibrium distribution of ubiquinone between the two sites. To examine such a possibility, we have used fluorescence quenching techniques which can resolve the location of certain molecules in the transverse plane of the membrane (Sikaris et al. 1981; Blatt et al. 1984).

Ubiquinone can quench the fluorescence of 12-(9-anthroyloxy) stearic acid (12-AS) in liposomes and mitochondrial membranes (Chance et al. 1975). The anthracene ring of this probe is centred approximately 15 Å from the membrane surface. A series of fluorescent fatty acids are now available which have the athroxyloxy group attached at different positions along the acyl chain, and numerous studies have shown that these can report the environment at a graded series of positions from the surface to the centre of the bilayer (Thulborn and Sawyer 1978;

Abbreviations: *n*-AS, n-(9-anthroyloxy) stearic acids (n = 6,9,12); *n*-AP, *n*-(9-anthroyloxy) palmitic acids (n = 2,16); *n*-AF, *n*-(9-anthroyloxy) fatty acids (n = 2,6,9,12,16); *n*-nitroxide stearic acids (n = 5,16); UQ_n, ubiquinone-*n* (n = 4,6,10); HBHM, heavy beef heart mitochondria

Tilley et al. 1979; Vincent et al. 1982). The 9, 12, and 16 derivatives of this series have been used to examine the effect of the isoprenoid chain length of ubiquinone on the thermotropic properties of dimyristoyl phosphatidylcholine bilayers (Katsikas and Quinn 1982).

Examination of model systems provides information on the basic physical chemistry of ubiquinone in synthetic phospholipid bilayers. There is evidence, however, that the characteristics of ubiquinone in mitochondrial membranes may be somewhat different. For example, the partition coefficient of ubiquinone into mitochondrial membranes is higher than into liposomes composed of egg yolk phosphatidylcholine (Degli Esposti et al. 1981). In addition, ubiquinone binding proteins are present in the mitochondrial membrane, although ubiquinone is present in a large molar excess over other electron carriers in the respiratory chain. For these reasons, we have chosen to conduct fluorescence quenching experiments with mitochondrial membranes rather than with synthetic lipid bilayers.

Materials and methods

n-(9-Anthroyloxy) fatty acids (*n*-AF; $n = 2, 6, 9, 12,$ and 16) were prepared by anhydride synthesis from the corresponding *n*-hydroxy fatty acids and anthracene-9-carboxylic acid (Thulborn and Sawyer 1978). Ubiquinones 4, 6, and 10 were a generous gift from Hoffman La Roche, Switzerland. *n*-(Doxyl) stearic acids ($n = 5, 16$) were purchased from Molecular Probes, Junction City, Oregon. All chemicals were of analytical grade.

Heavy beef heart mitochondria (HBHM) were prepared by the small scale method of Smith (1967). The medium used for the isolation and for all subsequent fluorescence spectroscopy was 10 mM *Tris*-HCl, 0.25 M sucrose, 0.2 mM EDTA, 1 mM succinate, pH 7.8. Rat liver mitochondria were prepared by the method of Johnson and Lardy (1967). Respiratory control and ADP/O ratios were measured polarographically (Estabrook 1967) using succinate and glutamate as substrates. For some experiments HBHM were lyophilized and extracted with *n*-pentane according to the method of Szarkowska (1966) to remove endogenous ubiquinone. Protein concentrations were determined by a Biuret method as modified by Jacobs et al. (1956).

Fluorescence probes were added as 10-µl aliquots of 1 mM stock solutions in methanol to 3 ml of mitochondrial suspension. Probe uptake was allowed to occur for 1 h at 4° C in the dark. Ubiquinones were prepared as 1-mM stock solutions in ethanol and stored at −20° C. The concentration was determined using $\Delta A^{275\,\mathrm{nm}}_{\text{ox-red}} = 12.5\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ (Degli Esposti et al. 1981). Ubiquinones were added as 10-µl aliquots to 2.5 ml of mitochondrial suspension followed by immediate mixing (Szarkowska 1966).

Steady state fluorescence and polarisation values were measured as described previously (Thulborn and Sawyer 1978). Excitation and emission wavelengths were 365 nm and 450 nm, respectively, and the corresponding slit widths were 4 nm and 6–10 nm. Cuvettes were thermostatted at 20° C. All intensities were corrected for dilution and light scattering. In the Cu^{2+} quenching experiments, no changes in light scattering were detected which could be attributed to Cu^{2+}-induced aggregation of membranes. Mitochondrial preparations used in these experiments had been washed twice in EDTA-free buffer to remove residual traces of the chelator.

Theory

Dynamic quenching of a membrane-bound fluorophore by a partitioning quencher can be described by the equation:

$$\frac{I_0}{I} - 1 = k_q \tau_F [Q'_M] \tag{1}$$

where I_0 and I are the intensities in the absence and presence of quencher, respectively, k_q is the bimolecular rate constant, τ_F is the lifetime of the fluorophore and $[Q'_M]$ is the concentration of quencher in the membrane expressed relative to the membrane volume. By defining a partition coefficient, $K_p = [Q'_M]/[Q'_A]$ where $[Q']$ is the quencher concentration in the aqueous phase, and taking into account the volumes of the two phases we obtain (Sikaris et al. 1981):

$$\frac{[Q_T]}{\frac{I_0}{I} - 1} = \frac{1}{k_q \tau_F} \frac{V_L}{V_T} + \frac{1}{k_q \tau_F K_{p,\text{local}}} \tag{2}$$

where $[Q_T]$ is the total concentration of quencher, V_L and V_T are the volume of lipid phase and the total volume, respectively, and $K_{p,\text{local}}$ is the local partition coefficient which describes the distribution of quencher between the aqueous phase and a subcompartment of the membrane phase. A plot of the left hand side of (2) versus V_L/V_T yields a straight line, and values of k_q and $K_{p,\text{local}}$ can be obtained from the slope and intercept when quenching occurs solely by a dynamic mechanism. For a mixture of static and dynamic quenching, the slopes and intercepts are dependent on $[Q_T]$. Extrapolation of slopes and

Table 1. Values of the relative absolute and local partition coefficients in native and pentane extracted mitochondria

Membrane	$K_{p,\,abs}$	$K_{p,\,local}$				
		2-AP	6-AS	9-AS	12-AS	16-AP
Native HBHM	33	300	33	120	380	> 3,000[a]
Pentane extracted HBHM	28	> 3,000[a]	31	131	256	88

[a] Lower limits of $K_{p,\,local}$. Accurate values could not be determined since the plots of (2) intersect the ordinate very close to the origin

intercepts to zero quencher concentration provides a value for $K_{p,local}$ (slope/intercept). This procedure is particularly appropriate for treating the quenching of *n*-AF by ubiquinone since this process is known to occur by a mixed dynamic-static mechanism (Chance et al. 1975).

An alternative treatment determines an absolute partition coefficient which describes the distribution of quencher between the aqueous and lipid compartments (Encinas and Lissi 1982). Quenching efficiency decreases as the membrane concentration increases and the quencher becomes diluted in the membrane phase. A given level of quenching corresponds to a characteristic average number of quenching molecules in the membrane ($\bar{n}$). At constant $\bar{n}$:

$$[Q]_T = \bar{n}[\text{Memb}] + [Q']_A\,, \tag{3}$$

where [Memb] is the membrane concentration. A plot of $[Q]_T$ versus [Memb] yields a straight line with slope $\bar{n}$ and intercept $[Q']_A$.

When the quencher exhibits a mixture of binding and partitioning behaviour (Haigh et al. 1978):

$$\bar{n} = K_{p,\text{abs}}\, v_M [Q']_A + \frac{pK_b[Q']_A}{1 + K_b[Q']_A}\,, \tag{4}$$

where $K_{p,abs}$ is the absolute partition coefficient, v_M is the volume of lipid in 1 mole of membranes, K_b is the binding constant and p is the number of binding sites. A plot of $\bar{n}$ versus $[Q']_A$ will yield a straight line at high quencher concentrations ($[Q']_A \gg 1/K_b$) with slope $K_{p,abs}\ v_M$ and intercept p.

A volume ratio (V_R) can now be defined which quantitatively describes the subcompartment of the membrane phase within which the quenching reactions are localized (Blatt et al. 1984).

$$V_R = \frac{K_{p,\text{abs}}}{K_{p,\text{local}}} = \frac{\text{quenching subvolume}}{\text{membrane volume}}\,. \tag{5}$$

When $V_R = 1$, quencher reactions occur within the total volume of the membrane. When $0 < V_R < 1$, the quenching reactions are confined to a subvolume within the membrane system. When the series of *n*-anthroyloxy fatty acids are used as fluorophores, the volume ratio provides information about the subvolume of the membrane within which quenching occurs, and an indication of the transverse position of quencher relative to the fluorophore.

In the case of mitochondria, the concentration of the membrane (mitochondrial membrane/unit volume) and the volume of the lipid phase required in (2)–(4) are difficult to calculate. The problem is overcome by expressing the membrane concentration in terms of the concentration of membrane protein, in which case the constants involved in the conversion of membrane protein to volume of the lipid phase (e.g., protein : lipid ratio, lipid density) cancel out in calculation of the volume ratio, V_R. For this reason the values of partition coefficients listed in Table 1 are relative values.

Results

Position of fluorescence probes in the mitochondrial membrane

The fluorescence of *n*-AF probes is enhanced when they are transferred from a polar to a non-polar environment and this phenomenon can be used to study their interaction with membranes (Haigh et al. 1978). Using this property, we found that uptake of 2-, 6-, 9-, and 12-AF (3.1–3.8 μM) was essentially complete within 1 h at 20° C when the concentration of mitochondrial protein was 0.1 mg/ml; that is, at this membrane concentration the amount of probe remaining in the aqueous phase was negligible (data not shown).

Fluorescence quenching experiments were designed to establish the relative transverse positions of the anthroyloxy groups of *n*-AF probes in the mitochondrial membrane. Figures 1a and b show the quenching of *n*-AF fluorescence by Cu^{2+} and 16-NS. Cu^{2+} quenches membrane bound fluorophores from the aqueous phase or by limited penetration of the bilayer (Thulborn and Sawyer 1978). Addition of Cu^{2+} leads to an order of quenching efficiency of

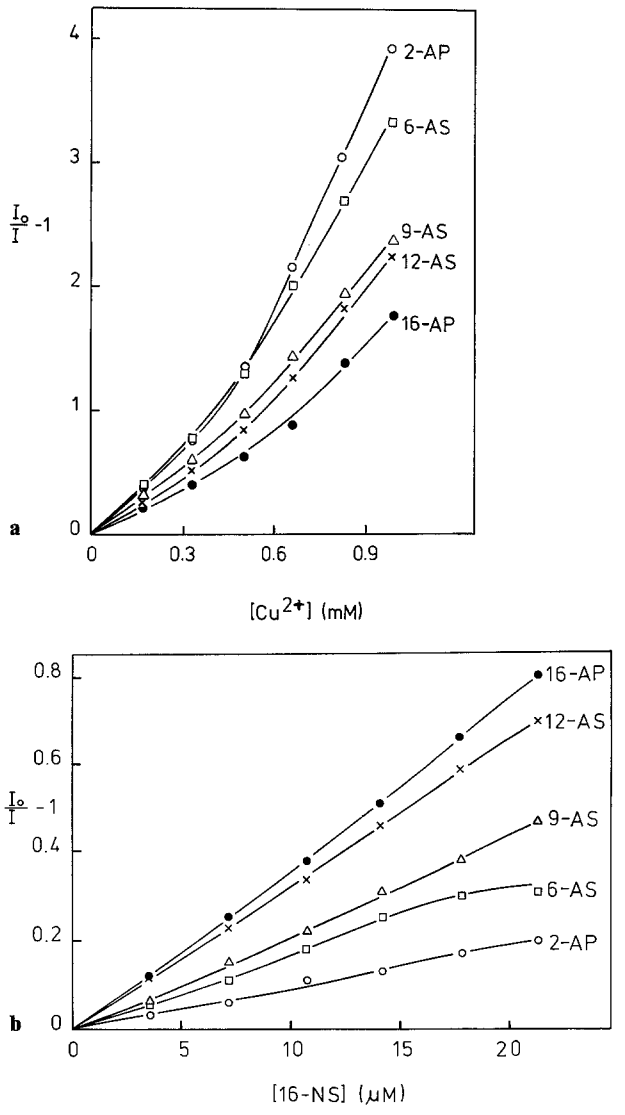

Fig. 1a and b. Stern-Volmer plots for the quenching of *n*-AF fluorescence in HBHM by (**a**) $CuSO_4$ and (**b**) 16-NS. [HBHM] = 0.1 mg/ml, [*n*-AF] = 3.1–3.8 μM

2- > 6- ~9- > 12- > 16-AF indicating that 2-AP is closest to the membrane surface and 16-AP is deepest in the membrane. The upward curving plots indicate a significant static component of quenching. However, the Stern-Volmer quenching constants (K_{sv}) determined from the initial slopes of Fig. 1a were within the range reported by Chalpin and Kleinfeld (1983) for AF probes in phosphatidylcholine vesicles and in vesicles derived from human erythrocyte membranes (10^2–10^3 M^{-1}). Addition of 16-NS, whose quenching moiety (a doxyl group) is probably located near the membrane centre, leads to the reverse order of quenching efficiency (Fig. 1b). 5-NS, whose doxyl group is probably at an intermediate depth in the membrane, quenches the probes in the order 6- > 9- ~12- > 2- > 16-AF (data not shown).

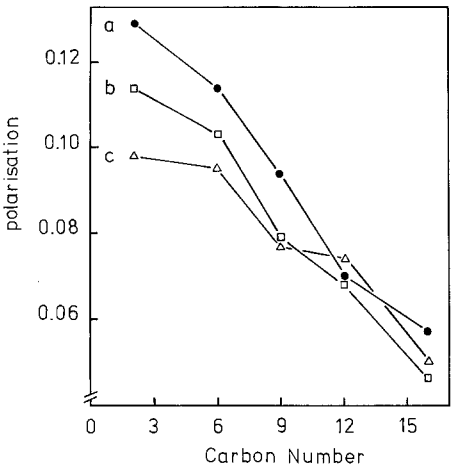

Fig. 2. Polarisation values of the n-AF probes in HBHM. Concentrations of mitochondrial protein are (a) 0.1 mg/ml, (b) 0.5 mg/ml, and (c) 1.0 mg/ml. [*n*-AF] as in Fig. 1

These trends are consistent with previous data which show that the anthroyloxy groups of the *n*-AF probes locate at a graded series of depths in the lipid bilayer (Thulborn and Sawyer 1978; Thulborn et al. 1979).

The fluorescence polarisation values of the *n*-AF probes in HBHM membranes are summarized in Fig. 2. The major feature of the data is the decrease in polarisation as the fluorophore is moved deeper into the membrane. Similar polarisation gradients have been observed in erythrocyte and hepatocyte membranes (Howard and Sawyer 1980), and in phospholipid vesicles (Thulborn et al. 1979; Kutchai et al. 1983), and reflect the gradient of membrane fluidity from the surface to the centre of the lipid bilayer. The decrease in polarisation with increasing membrane concentration shown in Fig. 2, an effect particularly apparent at the 2- and 6- position, is due to a light scattering artefact (Kutchai et al. 1982).

Kinetics of ubiquinone uptake

Figure 3 shows the change in fluorescence of 6-AS in mitochondrial membranes as a function of time after ubiquinone addition. When UQ_4, UQ_6, or UQ_{10} are added to native or pentane-extracted HBHM, the fluorescence reaches a constant level after ~10 min indicating that the system has reached equilibrium. In all subsequent experiments, the intensity was recorded 10 min after quencher addition. Quenching in the ubiquinone-depleted membranes was approximately twice that in native membranes.

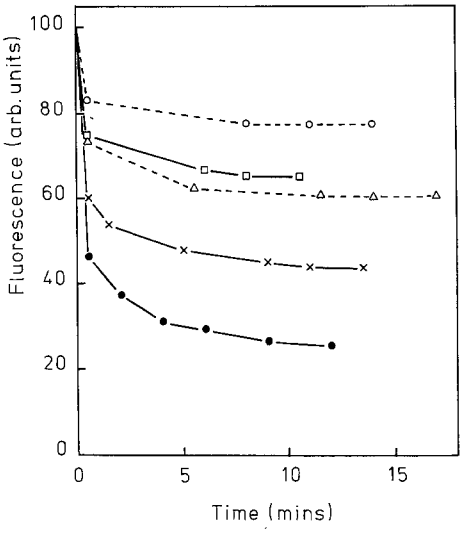

Fig. 3. Fluorescence of 6-AS in HBHM as a function of time after UQ addition. [HBHM] = 0.1 mg/ml. (○) and (□), [UQ-10] = 9.6 μM. (△) and (×), [UQ-6] = 13.7 μM. (○), [UQ-4] = 14.0 μM. The dashed lines indicate UQ was added to native HBHM and the continuous lines refer to pentane-extracted HBHM. [6–AS] = 3.8 μM

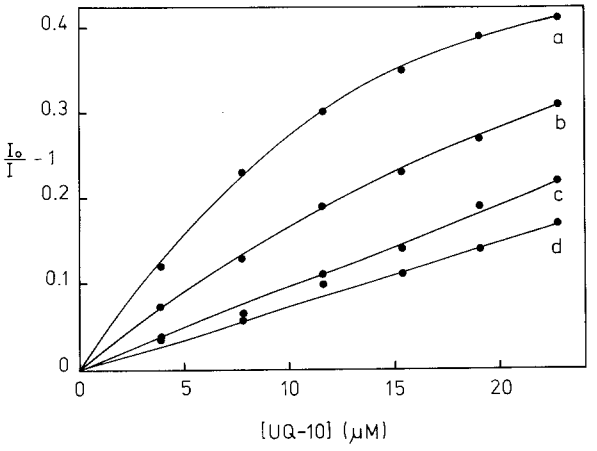

Fig. 4. Stern-Volmer plots for the quenching of 2-AP fluorescence by UQ-10 in HBHM. Concentrations of mitochondrial protein were (a) 0.05 mg/ml, (b) 0.1 mg/ml, (c) 0.15 mg/ml, and (d) 0.2 mg/ml. [2-AP] = 3.5 μM

Position of the benzoquinone ring

The quenching of 2-AP fluorescence by UQ_{10} in HBHM membranes is shown in Fig. 4. The strong dependence of the quenching efficiency on the membrane concentration indicates that the quencher preferentially associates with the membrane phase. These data were analysed according to (2) and (3) to yield values of $K_{p,\text{local}}$ and $K_{p,\text{abs}}$ from which a value of V_R was obtained (5). Repeating this analysis for each member of the *n*-AF series yielded values of the partition coefficients and V_R which are summarized in Table 1 and Fig. 5. V_R is small when 2-AP is the

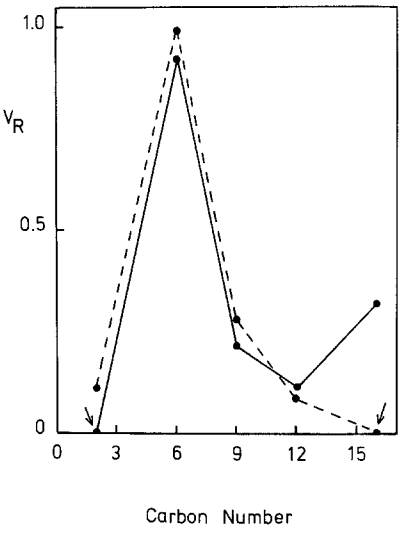

Fig. 5. A plot of the subvolume of the membrane in which the quenching interactions occur (V_R) against the carbon number of the *n*-AF probes. The dashed and continuous lines refer to native and pentane-extracted HBHM, respectively. The arrows indicate low values of V_R associated with high values of $K_{p,\text{local}}$ (see Table 1)

fluorophore, implying that the anthroyloxy group and the quinone are compartmentalized in a small fraction of the total membrane volume near the membrane surface. When 6-AS is the fluorophore, V_R is close to unity indicating that the fluorophore and quencher are interacting over the total volume of the membrane. As the fluorophore is moved deeper into the membrane (9-, 12-, and 16-AF), V_R decreases indicating that the fluorophore and quencher (benzoquinone) are again approaching each other. The data therefore indicate that the benzoquinone ring is distributed between two positions, one near the membrane surface and one close to the bilayer centre.

The above discussion relates only to oxidized ubiquinone and only to the position of the benzoquinone ring of that molecule rather than to the disposition of the isoprenoid side chain. The reduced form of ubiquinone does not quench the fluorescence of the anthroyloxy fatty acids (Chance et al. 1975).

Perturbation of the membrane

A valid criticism of the use of fluorescent probes for membrane studies is the possibility of perturbation of the lipid structure by the introduction of bulky fluorescent molecules. An estimate of such perturbation can be made by measuring the effect of the probe on the biological function of the membrane. The respiratory control ratio and ADP/O ratio of rat liver mitochondria was therefore measured in the

presence of fluorescent probes at levels similar to those used in the above experiments. The probes had no effect on the respiratory activity compared to the appropriate control.

Discussion

Most studies which have examined the position and orientation of ubiquinone in membranes have used model systems such as single bilayer vesicles and surface monolayers. The situation in the inner mitochondrial membrane is likely to be more complex, not only because of the heterogeneity of the phospholipid population, but also because of the presence of specific ubiquinone-binding proteins. Nevertheless, it is generally recognized that a substantial pool(s) of relative mobile ubiquinone is present in the mitochondrial membrane, and such a pool(s) is considered a necessary prerequisite for the protonmotive *Q*-cycle (Gutman 1980).

The use of the *n*-AF probes to establish the position of the benzoquinone ring assumes that the fluorophores themselves are positioned at a graded series of depths in the membrane. The quenching experiments summarized in Fig. 1a and b support this assumption. The quenching data cannot be explained on the basis of the variation in the lifetime of *n*-AF probes. Previous studies have shown that the lifetimes vary from about 10 ns for 2-AP to 13 ns for 12-AS (Thulborn et al. 1979) and would therefore favour stronger quenching of the deeper probes. Such is not the case for Cu^{2+} quenching (Fig. 1a). The lifetime variation could contribute to the order of quenching observed with 16-NS but it cannot account for it completely. For example, 16-AP would need to have *four times* the lifetime of 2-AP to account for the difference in quenching efficiency shown in Fig. 1b.

The gradient of polarisation values (Fig. 2) reflects the gradient in fluidity of the membrane lipid from the surface to the centre of the bilayer, and establishes that the probes report the characteristics of the lipid phase rather than characteristics of membrane proteins. It has been established previously that the *n*-AF probes are able to rotate almost isotropically in fluid membranes, and that the steady-state polarisation values reflect the kinetics of motion rather than bilayer order in the form of restricted probe motion (Vincent et al. 1982). The existence of a transverse fluidity gradient can lead to difficulties when interpreting fluorescence quenching data, because of the inverse relationship between the bimolecular quenching constant and the microviscosity. This highlights the importance of separating viscosity effects, as reflected in the bimolecular rate constant, from proximity effects as reflected in $K_{p,\,\mathrm{local}}$; this separation is accomplished by the analysis used in the present study.

The non-linearity of the Stern-Volmer plot observed at low lipid concentrations (Fig. 4) could have several origins. It could be due to two or more populations of fluorophores of different lifetime. It could be due to the binding of quencher to saturatable sites on or in the membrane (Thulborn and Sawyer 1978). It could also be due to phase separation or aggregation of UQ_{10} leading to a decrease in its effectiveness as a quencher. The analysis of $K_{p,\mathrm{abs}}$ is not affected by any such behaviour (Encinas and Lissi 1982), and it is likely that the analysis of $K_{p,\,\mathrm{local}}$ will be affected to only a minor degree since the values of $K_{p,\mathrm{local}}$ are extrapolated to zero concentration of quencher (Sikaris et al. 1981).

The existence of two transverse positions of the benzoquinone ring as revealed by the V_R analysis in Fig. 4, is relevant to proposals that ubiquinone exists in the inner mitochondrial membrane in more than one spatially compartmentalised pool. Analysis of oxidation-reduction kinetics led Kroger and Klingenberg (1973) to postulate the existence of two ubiquinone pools, one corresponding to 80%–90% of the total ubiquinone and being redox-active and kinetically homogenous, and the other being a relatively inactive pool. Our method of analysis does not allow us to define the fraction of quinone at each transverse site. Nevertheless, the two site model qualitatively explains the observation of two kinetically distinct pools in terms of spatial compartmentation of the benzoquinone ring. The interchange of ubiquinone between the active and inactive pools must be slow or nonexistent in order to explain the kinetic data. Such interchange must also be slower than the nanosecond time scale on which the fluorescent quenching occurs. The *n*-AF probes have lifetimes in the range 8-14 ns. A longer time scale would be required to sense the exchange of ubiquinone between the two transverse pools.

Studies of ubiquinone in monomolecular films have suggested that at low surface pressures the isoprenoid chain is situated in the region occupied by the phospholipid acyl chains (Quinn and Esfahani 1980). As the surface pressure is increased to levels thought to exist in natural membranes, the ubiquinone is forced out of the monolayer into a separate phase which overlays the monolayer surface. It is possible, therefore, that ubiquinone is 'energetically poised' between these two locations which may correspond to the sites detected by the fluorescent analysis described in this paper. It would seem unlikely that either pool represents ubiquinone bound to membrane enzymes, not only because of the

excess of ubiquinone over these proteins, but also because such ubiquinone would be largely immobile and unable to contribute to quenching of the probes which is known to occur substantially by a dynamic mechanism (Chance et al. 1975).

The extent to which the outer membrane of the mitochondrion might act as a reservoir or compartment for exogenous ubiquinone added to the system is unknown. However, because of the invaginations of the inner membrane into the mitochondrial matrix, the outer membrane represents only a small proportion of the total and is unlikly to represent a major compartment within which quenching can occur.

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