Determination of Partition and Lateral Diffusion Coefficients of Ubiquinones by Fluorescence Quenching of *n*-(9-Anthroyloxy)stearic Acids in Phospholipid Vesicles and Mitochondrial Membranes†

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ABSTRACT: The quenching of fluorescence of *n*-(9-anthroyloxy)stearic acids and other probes by different ubiquinone homologues and analogues has been exploited to assess the localization and lateral mobility of the quinones in lipid bilayers of model and mitochondrial membranes. The true bimolecular collisional quenching constants in the lipids together with the lipid/water partition coefficients were obtained from Stern–Volmer plots at different membrane concentrations. A monomeric localization of the quinone in the phospholipid bilayer is suggested for the short side-chain ubiquinone homologues and for the longer derivatives when cosonicated with the phospholipids. The diffusion coefficients of the ubiquinones, calculated from the quenching constants either in three dimensions or in two dimensions, are in the range of (1–6) × 10^{-6} cm^2 s^{-1}, both in phospholipid vesicles and in mitochondrial membranes. A careful analysis of different possible locations of ubiquinones in the phospholipid bilayer, accounting for the calculated diffusion coefficients and the viscosities derived therefrom, strongly suggests that the ubiquinone 10 molecule is located within the lipid bilayer with the quinone ring preferentially adjacent to the polar head groups of the phospholipids and the hydrophobic tail largely accommodated in the bilayer midplane. The steady-state rates of either ubiquinol 1–cytochrome *c* reductase or NADH:ubiquinone 1 reductase are proportional to the concentration of the quinol or quinone substrate in the membrane. The second-order rate constants appear to be at least 3 orders of magnitude lower than the second-order constants for quenching of the fluorescent probes; this is taken as a clear indication that ubiquinone diffusion is not the rate-determining step in the quinone–enzyme interaction. Moreover, calculations of the lateral displacement rates for random walk in either long or short paths from the diffusion coefficients show that the diffusion of ubiquinone cannot be rate limiting in the integrated electron transfer from dehydrogenases to the cytochrome bc_1 complex. The decreased rate of electron transfer as a consequence of ubiquinone dilution by phospholipid enrichment of mitochondrial membranes [cf. Schneider, H., Lemasters, J. J., & Hackenbrock, C. R. (1982) *J. Biol. Chem. 257*, 10789–10793] is ascribed to the ubiquinone concentration being decreased below the Michaelis constant for interaction with its partner enzymes.

The observation that ubiquinone (Q)[1] in the inner mitochondrial membrane and in bacterial membranes and plastoquinone in the chloroplast thylakoid membrane are present in excess over the other electron-transfer components has prompted the idea that they function as a mobile carrier linking multiprotein redox complexes randomly distributed in the lipid bilayer by rapid diffusion (Green, 1966). From a kinetic analysis of the rate of electron input to ubiquinone (NADH:Q reductase and succinate:Q reductase) and of electron output from reduced ubiquinone (ubiquinol oxidase), under a wide range of input and output rates (V_{red} and V_{ox}, respectively), Kröger et al. (1973a,b) established that ubiquinone in mitochondria exists as a homogeneous mobile pool, shuttling electrons from each dehydrogenase molecule to each molecule of the enzyme oxidizing the quinol (ubiquinol–cytochrome *c* reductase). The overall observed rate through the respiratory chain (V_{obsd}) follows the relation

$$V_{obsd} = V_{red}V_{ox}/(V_{red} + V_{ox}) \quad (1)$$

This expression, known as the homogeneous pool equation, states that the current through the quinone pool is determined by the combination of electron influx and efflux. Such behavior has been confirmed in a large variety of experimental systems [cf. Gutman (1985)].

Ragan and co-workers (Ragan & Heron, 1978; Heron et al., 1978), studying the interaction of isolated complex I (NADH:Q reductase) with isolated complex III (ubiquinol–cytochrome *c* reductase), confirmed such pool behavior in the presence of added phospholipids but showed that in lipid-depleted preparations the pool behavior was lost and that interaction with the complexes appeared to be stoichiometric. As an explanation, they offered an alternative picture, where diffusion and collision of protein complexes, each carrying

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[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; *n*-AS, *n*-(9-anthroyloxy)stearic acid; bc_1 complex, cytochrome bc_1 complex (complex III, ubiquinol–cytochrome *c* reductase); cmc, critical micelle concentration; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; DML, dimyristoyllecithin; DPH, 1,6-diphenyl-1,3,5-hexatriene; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; FRAP, fluorescence recovery after photobleaching; NADH, reduced nicotinamide adenine dinucleotide; NBQ, 5-methyl-6-nonyl-1,4-benzoquinone; NMR, nuclear magnetic resonance; 5-NS, 5-(*N*-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid; 16-NS, 16-(*N*-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid; PL, phospholipids; Q, ubiquinone; QH_2, ubiquinol; SBQ, 2,3-dimethoxy-5-methyl-6-(10-sulfodecyl)-1,4-benzoquinone; Tris, tris(hydroxymethyl)aminomethane.

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bound ubiquinone, were responsible for the pool behavior. Lipid depletion, however, promoted fixed associations between complexes, eliciting a different, stoichiometric, behavior. The presence of "Q-binding proteins" in electron-transfer complexes (Nishi et al., 1979; Yu & Yu, 1980; King, 1982) has been considered in line with this reasoning. An alternative explanation for the loss of the pool behavior has been offered by Gutman (1985), who suggested that diffusion of ubiquinone becomes rate limiting in the lipid-depleted complexes. Such a "solid-state" behavior (Rich, 1984) fails to explain, however, why a fixed 1:1 stoichiometry should be reached (Ragan & Cottingham, 1985).

A different approach to the demonstration of pool behavior was employed by Hackenbrock and his co-workers (Schneider et al., 1980, 1982). By increasing the phospholipid content of mitochondrial membranes, they observed an inhibition of electron flow between the enzymes reducing and oxidizing the ubiquinone, an inhibition that was reversed by adding ubiquinones together with the phospholipids.

This observation was taken to mean that the rate of electron transfer between dehydrogenases and cytochromes is diffusion-limited and that diffusion of ubiquinone is the rate-controlling step in the overall process (Hauska & Hurt, 1982). The same reasoning was applied to similar results in phospholipid-enriched chromatophores from *Rhodopseudomonas sphaeroides* (Casadio et al., 1984), although processes influenced by the quinone concentration, rather than by Q diffusion directly, were considered likely to determine the rate-limiting step of electron transfer. Likewise, the second-order reduction of cytochrome *b*-561 in chromatophores by the quinol produced by photoreduction in the reaction center (Crofts & Wraight, 1983; Snozzi & Crofts, 1984) was used to calculate a diffusion coefficient $D = 10^{-10}$ cm^2 s^{-1}, assuming lateral diffusion was rate-limiting. Crofts and Wraight (1983) discussed the possibility that the rate-limiting steps were quinol release from the reaction center and its binding to the bc_1 complex but concluded that these steps should constitute only a minor contribution to the second-order rate constant for the reaction.

On the other hand, in the case of the chloroplast thylakoid membranes, the diffusion properties of plastoquinone in the membrane appear to be critical for the overall rate of electron flow (Haehnel, 1984). 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, much of which is located in the exposed areas close to photosystem I. A very high lateral diffusion rate is required for such processes, with D in the range of 10^{-6} cm^2 s^{-1} or more (Millner & Barber, 1984).

Much of the uncertainty still existing on the mechanism of electron transfer in the ubiquinone (and plastoquinone) region of electron-transfer chains could be clarified by direct measurements of the lateral diffusion coefficients of the quinones in the membranes; however, in spite of their physiological relevance, there have as yet been only two studies dealing with direct measurements of the lateral diffusion coefficients of ubiquinones. Gupte et al. (1984), using a fluorescent derivative of 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)benzoquinone and the method of fluorescence recovery after photobleaching (FRAP), reported D values on the order of 10^{-9} cm^2 s^{-1}. On the other hand, in a previous report (Fato et al., 1985) we have approached the problem using *natural* ubiquinones and exploiting the fact that in their oxidized form they quench the fluorescence of several fluorophores by a collisional mechanism. The lateral diffusion coefficients of ubiquinone homologues calculated by this method were in the order of 10^{-6} cm^2 s^{-1} in phospholipid vesicles.

In this paper we report a detailed analysis of the results obtained in model systems and extended to bovine heart submitochondrial particles.

Experimental Procedures

Materials. The ubiquinone homologues were kind gifts from Eisai Co., Tokyo, Japan; they were stored as solutions in absolute ethanol at −20 °C at concentrations ranging between 5 and 35 mM as determined spectrophotometrically at 275 nm with an average extinction coefficient of 12.5 mM^{-1} cm^{-1} (Mayer & Isler, 1971). The ubiquinone analogues 2,3-dimethoxy-5-methyl-6-nonyl-1,4-benzoquinone (NBQ) and 2,3-dimethoxy-5-methyl-6-(10-sulfodecyl)-1,4-benzoquinone (SBQ) were kind gifts of Professor G. von Jagow, University of Munich, FRG. When necessary, ubiquinones were reduced according to the method of Rieske (1967) and stored in absolute ethanol as described by Degli Esposti et al. (1981a).

Mixed soybean phospholipids (asolectin) were purchased from Associated Concentrates Inc., New York, and purified according to Kagawa and Racker (1971) to remove neutral lipids. Dimyristoyllecithin was purchased from Sigma, St. Louis, MO, and cholesterol from British Drug House, Poole, U.K.; egg lecithin was purified from egg yolks according to Robinson (1960). The fluorescent probes 3-, 6-, and 12-(9-anthroyloxy)stearic acids (AS) were obtained from Molecular Probes, Junction City, OR, and stored as solutions in absolute ethanol at −20 °C at a concentration of 1 mM. 1,6-Diphenyl-1,3,5-hexatriene (DPH) from Fluka, Buchs, Switzerland, and 1-anilinonaphthalene-8-sulfonate (ANS) from Sigma, recrystallized from water, were also stored as solutions in absolute ethanol. The spin-labels 5-(*N*-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid (5-NS) and 16-(*N*-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid (16-NS), purchased from Syva Inc., Palo Alto, CA, were stored in ethanol at −20 °C at a concentration of 10 mM.

Preparative Procedures. Phospholipid vesicles were prepared from dry phospholipids by sonic irradiation in a MSE sonifier for 40 min under nitrogen flux as described by Degli Esposti et al. (1981a). The vesicles were unilamellar with an average diameter of 350–400 Å, as controlled by electron microscopy. For preparation of mixed vesicles of phospholipids and ubiquinones, the compounds in chloroform–methanol (2:1 by volume) were evaporated together at the required ratios under nitrogen, resuspended, and sonicated as above. A similar procedure was used to prepare vesicles of phospholipids and cholesterol (molar ratio 2:1). The final concentrations of the suspensions were 4–12 mg of phospholipids/mL as detected by the phosphorus content (Marinetti, 1962); the suspensions were used within 2 days after preparation.

Bovine heart mitochondria were prepared by the procedure of Smith (1967) and submitochondrial particles (EDTA–particles) according to Lee and Ernster (1968).

The cytochrome bc_1 complex (ubiquinol–cytochrome *c* reductase, EC 1.10.2.2) was purified from mitochondria by the procedure of Rieske (1967), and complex I (NADH:ubiquinone oxidoreductase, EC 1.6.99.3) was prepared by the method of Hatefi and Rieske (1967).

Vesicles containing either the bc_1 complex or complex I with different amounts of phospholipids were prepared by cholate dialysis [cf. Degli Esposti et al. (1983)]. Protein was assayed by a biuret method (Gornall et al., 1949).

Enzymatic Assays. NADH:ubiquinone 1 oxidoreductase activity was assayed spectrophotometrically as described elsewhere (Cabrini et al., 1981) and ubiquinol–cytochrome

c oxidoreductase activity by the method of Rieske (1967) as modified by Degli Esposti and Lenaz (1982a). The spectrophotometric assays were accomplished by using a Sigma-Biochem dual-wavelength spectrophotometer equipped with a rapid mixing apparatus of our own design, with a resolution time of ca. 100 ms.

Fluorescence Studies. The fluorescent probes were incorporated into the lipid vesicles by addition of the ethanolic solution to an aqueous suspension of vesicles in 10 mM Tris-HCl buffer, pH 7.5, at a probe:phospholipid molar ratio of 1:150, and then the mixture was incubated for 40 min at room temperature; longer incubations did not result in any further enhancement of fluorescence, indicating that maximal incorporation of the probe was achieved. Fluorescence studies were carried out with a Perkin-Elmer MPF-4 spectrofluorometer; the excitation wavelength was 366 nm for *n*-AS, 356 for DPH, and 350 nm for ANS; the fluorescence spectra were recorded in all cases, and the emission value at the maximum was used for each experimental point. The light scattering was usually negligible, as demonstrated by blank assays were the probe was omitted; when necessary, the light scattering was subtracted from the emission spectra.

Ubiquinones were incorporated into lipid vesicles by addition of different amounts of an ethanolic solution of quinones or by cosonication with the phospholipids (see above). The same procedure was applied for Q addition to EDTA–particles. The total concentration of ethanol never exceeded 5 μL/mL and was previously found to induce no structural or fluidity alteration of the lipid vesicles (Spisni et al., 1978). When ubiquinones were added from ethanolic solutions, control assays were made where the quinones and the probes were mixed in the buffer in the absence of phospholipids; a slight fluorescence of the anthroylstearate probes was observed under such conditions, probably reflecting micellization of the fatty acid derivatives; such fluorescence was usually less than 5% of the total fluorescence obtained in the presence of phospholipid vesicles.

The spin probes 5-NS and 16-NS were incorporated into phospholipid vesicles by addition of the ethanolic solution to the suspension.

Fluorescence polarization was determined under the conditions described by Shinitzky et al. (1971) and with the same instrument as above equipped with polarization accessories.

Partition Coefficients of Ubiquinones in Phospholipid Vesicles. The partition of ubiquinones in the phospholipids was investigated independently of the fluorescence-quenching method (see below) by the procedure of Degli Esposti et al. (1981a) or from quinone solubility (Ragan, 1978) according to the equation (Hill, 1974)

$$PS = 2 \tag{2}$$

where P is the partition coefficient in (moles of Q per mole of PL)/(moles of Q per mole of water) and S is the solubility in moles of Q per mole of water.

For the homologues Q_2 and Q_3, solubility was measured by evaluating a critical micelle concentration (cmc); the cmc was determined by the red shift and the decrease of the extinction coefficient accompanying aggregation of ubiquinone or ubiquinol homologues in aqueous media (Degli Esposti et al., 1981a,b; Lenaz & Degli Esposti, 1985). The spectrophotometric determinations were performed in a Perkin-Elmer 559 UV–vis spectrophotometer.

Calculation of Lateral Diffusion from Fluorescence Quenching. Oxidized ubiquinones quench the fluorescence of AS derivatives largely by a collisional mechanism (Chance et al., 1975). A similar collisional mechanism has been proven for the other probes used, as shown by the linearity of plots of I_0/I vs. Q concentration, according to the Stern–Volmer relation

$$I_0/I = 1 + k\tau_0[Q] \tag{3}$$

where I_0 is the fluorescence intensity in the absence of quencher, I is the fluorescence intensity in the presence of quencher concentration [Q] in moles per liter, τ_0 is the fluorescence lifetime, in seconds, in the absence of quencher, and k is the bimolecular quenching constant expressed in M^{-1} s^{-1}. Deviations from linear Stern–Volmer plots in organic solvents were observed only at high ubiquinone concentrations, indicating that other quenching mechanisms may become operative.

When the probes are incorporated into phospholipid vesicles, the quenching by quinones also follows a Stern–Volmer relationship, except at high quinone/phospholipid ratios. Each point in the plots was the average of three separate determinations, and in most cases the points fitted straight lines with high reproducibility. Occasionally the plots were concave upward, indicating the presence of static quenching. Correction for static quenching, when necessary, was usually accomplished by extrapolating the slope from the values at lower quinone concentrations. Controls evaluating directly the dynamic component of quenching from dynamic fluorescence measurements of the fluorescence lifetimes at different quencher concentrations revealed that the static component (Lakowicz, 1983) was negligible and that the empirical corrections were suitable under our experimental conditions.

The fluorescence lifetimes were determined by time-resolved fluorescence spectroscopy using the single-photon time-correlating technique. The experiments were kindly performed by Drs. S. Dellonte and F. Barigelletti of the Institute of Photochemistry of the University of Bologna under the conditions described by Gardini et al. (1980); the decay curves were analyzed by iterative deconvolution dealt with by the nonlinear least-squares process of Grinwald and Steinberg (1974). The fluorescence lifetime of 12-AS in asolectin vesicles in the absence of quenchers was found to be 9.4 ns at 25 °C, in fair agreement with that reported by Thulborn et al. (1979).

Experiments using deoxygenated solutions showed that the presence of oxygen had no appreciable effect on the quenching constants of the ubiquinones, either in organic solvents or in lipid bilayers.

The extent of collisional quenching in a lipid bilayer depends upon the lipid/water partition coefficient and upon the rate of diffusion of the colliding species in the lipid bilayer (Lakowicz & Hogen, 1980). Therefore, the collisional quenching of fluorescence can be used for calculating the partition and diffusion coefficients for different ubiquinone homologues in lipid vesicles. In a membrane, where quenching occurs only in the lipid phase and partition with the water phase (or with any environment where quenching does not occur) may be significant, the Stern–Volmer relation for collisional quenching is modified, and the following relation holds:

$$\frac{1}{k_{app}} = \alpha_m\left(\frac{1}{k_m} - \frac{1}{k_m P}\right) + \frac{1}{k_m P} \tag{4}$$

where k_{app} is the apparent (measured) bimolecular quenching constant in M^{-1} s^{-1}, α_m is the volume fraction of the membrane, k_m is the biomolecular quenching constant in the membrane phase, and P is the partition coefficient, in units of (moles of Q per liter of PL)/(moles of Q per liter of water). The partition coefficients have been usually transformed and ex-

pressed as mole fractional ratios, i.e., (moles of Q per mole of PL)/(moles of Q per mole of water).

A plot of $1/k_{app}$ as a function of α_m gives a straight line with $1/k_mP$ as intercept and $(1/k_m - 1/k_mP)$ as slope. The correlation coefficient was usually ≥ 0.95.

In practice, when ubiquinones were added from ethanolic solutions, the experiments were made by adding progressive amounts of quinones to different sets of phospholipid concentrations [usually from 0.25 to 5 mg/mL, corresponding to α_m values of $(0.25–5) \times 10^{-3}$, on the assumption that 1 mg of phospholipid occupies 1 μL; cf. Lakowicz & Hogen (1980)]. When the Q homologues were added by cosonication, 24 experimental sets were prepared, corresponding to six ubiquinone concentrations from 0 to 36 μM and four phospholipid concentrations from 0.25 to 2 mg/mL.

From the values of k_m, diffusion coefficients were calculated routinely by the equation of Smoluchowski as modified for fluorescence encounters (Lakowicz & Hogen, 1980):

$$k_m = 4\pi\gamma R_{pq} N'(D_p + D_q) \quad (5)$$

where γ is the quenching efficiency of the fraction of collisional encounters that are effective in quenching, R_{pq} is the sum of the molecular radii of probe plus quencher, N' is Avogadro's number per millimole, and D_p and D_q are the diffusion coefficients of the probe and quencher, respectively, in the membrane, in units of $cm^2\ s^{-1}$. Since it has not been possible to measure the quenching efficiency of the ubiquinones on the AS probes in organic solvents (cf. Results), we have assumed that the quenching efficiency would be 1; thus the diffusion coefficients calculated in this way are the smallest possible.

According to Hardt (1979), the dimensionality of the diffusion may markedly affect the rate of diffusion-controlled reactions; with a different approach, an equation was derived for diffusion in two dimensions, in which the apparent second-order collisional constant becomes concentration dependent.

By using the above relations, it was possible to calculate only the sum of the diffusion coefficients of the probe plus quencher; we have therefore measured the diffusion of the probe independently using as quencher the spin-labels 5-NS or 16-NS (Bieri & Wallach, 1975); these molecules are very similar to the AS derivatives, so they must have comparable diffusion coefficients. The values found for AS + NS diffusion have been divided by two and subtracted from the values of diffusion of AS plus quinones.

EPR Spectroscopy. The microviscosity of lipid bilayers was investigated by determining the rotational motion of the lipid-soluble spin-label 16-NS. The apparent rotational correlation time τ_c was calculated from the EPR spectra by using the Kivelson relation (Kivelson, 1960). EPR spectra were recorded in a Varian E4 spectrometer at room temperature (microwave frequency, 9.52 GHz; receiver gain, 4×10^3; modulation amplitude, 2 G; time constant, 0.5 s). Other conditions were as described by Fato et al. (1984).

The membrane viscosity was calculated from the correlation times (Seelig & Seelig, 1980), assuming a rotational radius for 16-NS of 4 Å (Dix et al., 1978).

Results

Quenching of 12-AS Fluorescence by Ubiquinones. Different ubiquinone homologues (from Q_0 to Q_{10}) in their oxidized forms quench the fluorescence of either DPH or *n*-AS in organic solvents; the extent of quenching in a given solvent is very similar for all ubiquinone homologues tested, with a trend to smaller constants for bulkier long-chain ubiquinones. The quenching follows a Stern–Volmer relationship and is therefore largely collisional. The absorption spectra of DPH and 12-AS are unchanged upon Q_0 addition, a further indication of the prevalent collisional nature of the quenching.

Experiments designed to calculate the quenching efficiency γ (the fraction of the collisional encounters that are effective in quenching) were performed in organic solvents of known or measurable viscosity and by applying the Smoluchowski equation (eq 5) as described by Lakowicz and Hogen (1980); the diffusion coefficients D calculated from the Stokes–Einstein relation may be underestimated for molecules having radii comparable to that of the solvent; we have therefore used a value of $D = 10^{-5}\ cm^2\ s^{-1}$ as given by Marcus and Hawley (1970) for α-tocopherol in acetonitrile. By application of this procedure, efficiencies γ were usually found to be higher than 1.

There may be several reasons for the high experimental quenching efficiencies. One reason may be in the inappropriate use of the dimensions of the probe and/or the quencher molecules in tridimensional solutions; many of the molecules used are not spherical, and the correct radius to feed into the Smoluchowski equation is questionable. Another reason might be in the formation of mixed micellar aggregates of some of the molecules in organic solvents; the coexistence of probe and quencher molecules in the same clusters would greatly enhance the frequency of collisional encounters by the proximity effects.

Stern–Volmer plots of τ_0/τ, eliminating the static component of quenching, using Q_0 and DPH in cyclohexane to eliminate the possibility of micellar aggregates, resulted in bimolecular quenching constants of $3 \times 10^{10}\ M^{-1}\ s^{-1}$, with γ calculated to be 1.5; this is close to the limit of error established by Lakowicz and Hogen (1980) for calculating quenching efficiencies.

The uncertainty of measuring reliable quenching efficiencies for the AS derivatives has led us to assume it equal to 1; thus the diffusion coefficients calculated by the Smoluchowski equation in the lipid bilayers (see later) are the smallest possible and would be higher for $\gamma < 1$.

Incorporation of short isoprenoid chain ubiquinone homologues into phospholipid bilayers was achieved by simple incubation with the homologues added as ethanolic solutions. Stern–Volmer plots of quenching of 12-AS fluorescence by the quinones are usually linear [cf. Fato et al. (1985)], indicating the prevalent collisional nature of the quenching also in the lipid bilayers. Deviations from linearity are usually present at low membrane fractional volumes and/or high quinone concentrations; when present, such deviations are relatively small and have been corrected (cf. Experimental Procedures).

When in the phospholipids, quinones partition in the hydrophobic membrane interior, as previously established by spectrophotometric techniques (Degli Esposti et al., 1981a), although their location within the lipid bilayer is still debated (Lenaz & Degli Esposti, 1985; Lenaz et al., 1984; Kingsley & Feigenson, 1981; Stidham et al., 1984; Quinn & Katsikas, 1985; Chatelier & Sawyer, 1985; Ulrich et al., 1985; Trumpower, 1981).

The possibility that high quenching could artifactually arise from location of both quinone and probe molecules in the same small aggregates in the water phase has been dismissed by several lines of experimental evidence. Control determinations clearly established that the fluorescence of 12-AS in the absence of phospholipids is very low, even if it is quenched by ubiquinone homologues; subtraction of the low basal fluorescence levels from the fluorescence in the presence of

phospholipids does not significantly affect the calculations, and the same values are obtained with and without the corrections. Futhermore, the fluorescence of DPH in buffer is negligible and is not quenched by Q_1; nevertheless, quenching of DPH fluorescence by Q_1 in the lipid phase is of the same order of magnitude as that obtained by 12-AS fluorescence in the presence of any of the Q homologues.

The monomeric random dispersion of the Q homologues together with the probe in the lipid bilayer is a prerequisite for quenching being a measure of unrestrained lateral diffusion within the bilayer. We consider it extremely unlikely that the quenching takes place within molecular clusters in the membrane, since dilution of the probe from 1:150 to 1:13 000 probe:phospholipid molar ratio does not affect the quenching constants. Furthermore, we have followed the procedure of Bieri and Wallach (1975) to clarify whether the quenching process might take place within molecular clusters in the membrane; in such a case quenching would not be proportional to quencher concentration but rather to the square root of quencher plus probe concentration. Modified Stern–Volmer plots for 16-NS yielded critical concentration values of 0.06 (i.e., 6 probe *plus* quencher molecules per 100 phospholipid molecules), close to the value of 0.04 calculated by Bieri and Wallach (1975) for 16-NS in fluid bilayers. For ubiquinones, quenching does not vary linearly with $(c + 1/c)^{1/2}$, indicating that the quinone and probe molecules are free to diffuse laterally. We are therefore convinced, under our experimental conditions, that physiological concentrations of ubiquinones do not aggregate together with the probes and hence do not induce an artificial enhancement of the apparent bimolecular quenching constants. Furthermore, aggregation of quencher and probe is very unlikely with small hydrophobic molecules such as Q_1 and DPH; however, the quenching with these compounds is actually *higher*, as one should expect for molecules of smaller size.

From plots of $1/k_{app}$, as obtained from the primary Stern–Volmer plots, vs. α_m, the values for the partition coefficient P and the true bimolecular quenching constant in the membrane k_m have been obtained [cf. Fato et al. (1985)]. The partition coefficient, expressed in molar units, can be converted into fractional mole units (moles of Q per mole of PL)/(moles of Q per mole of water).

Figure 1 shows Stern–Volmer plots of 12-AS fluorescence quenching by Q_3 by plotting τ_0/τ vs. [Q]; the lifetime values, obtained by time-resolved fluorescence measurements, express collisional quenching uniquely (Lakowicz, 1983). The P and k_m values obtained were 1.1×10^4 (on a mole fraction basis) and 4.6×10^9 M^{-1} s^{-1}, respectively, in fair agreement with the data obtained from fluorescence intensity measurements, showing an average k_m of $(6.4 \pm 1.8) \times 10^9$ M^{-1} s^{-1} from nine separate determinations.

From the fluorescence measurements it was observed that the manner of addition of the quinones to the lipids strongly modifies the quenching characteristics; in particular, the long-chain homologues are not able to quench 12-AS fluorescence when added from ethanolic solutions [in accordance with the results of Quinn and Katsikas (1985)] but induce consistent quenching when cosonicated with the phospholipids.

Table I reports the partition coefficients and k_m values obtained for different ubiquinone homologues and analogues and for the probes 16-NS and 5-NS. The k_m for the nitroxide derivatives is lower than for the ubiquinone homologues, suggesting a different location in the membrane; signficantly, the k_m for the sulfate ubiquinone analogue SBQ is lower than

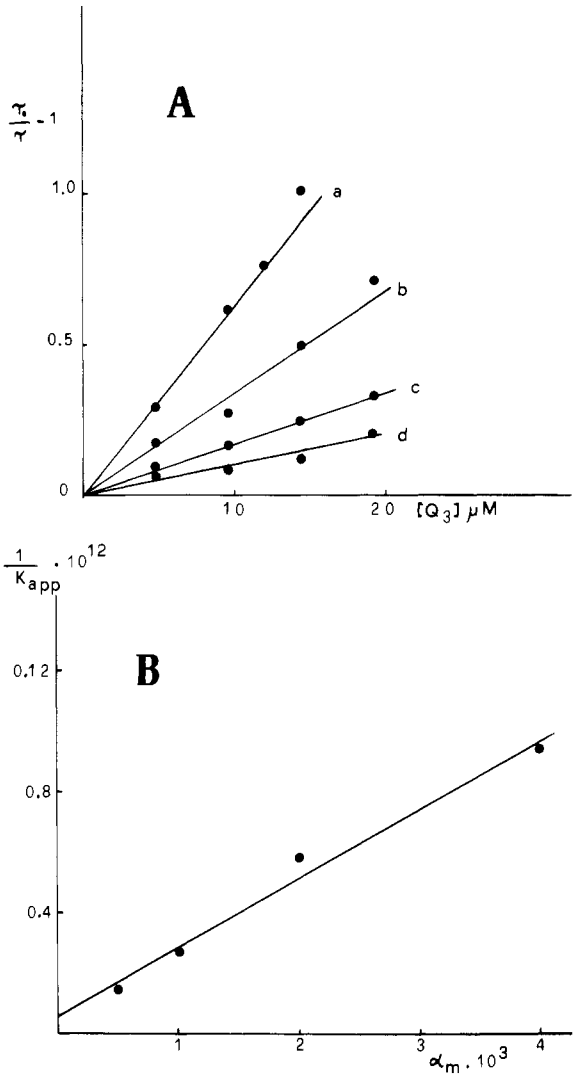

FIGURE 1: (A) Stern–Volmer plots of quenching of 12-AS fluorescence at 25 °C by Q_3 in phospholipid vesicles at different fractional volumes, as observed by experimentally determined lifetimes (see text). The probe to phospholipid ratio was kept 1:150 (mol:mol). The α_m were (a) 0.5×10^{-3}, (b) 1×10^{-3}, (c) 2×10^{-3}, and (d) 4×10^{-3}. (B) Plot of $1/k_{app}$ vs. α_m using the values from panel A. The line derived from linear regression analysis had a correlation coefficient of 0.98.

Table I: Quenching Constants and Partition Coefficients for Different Q Homologues in Asolectin Bilayers at 25 °C Measured by Quenching of Fluorescence of 12-AS

ubiquinone homologue or analogue	method of addition	$k_m \times 10^{-9}$ (M^{-1} s^{-1})	$P \times 10^{-4}$ [(mol of Q/mol of PL)/(mol of Q/mol of water)]
Q_1	added	3.3	9.1
Q_2	added	5.9	17.6
Q_3	added	6.4	18.5
Q_7	added	0.6	0.6
Q_7	cosonicated	3.9	3.2
Q_{10}	cosonicated	2.3	6.0
DB	added	6.7	8.4
NBQ	added	5.5	6.9
SBQ	added	2.0	1.8
16-NS	added	1.0	60.9
5-NS	added	0.32	5.9

that for the corresponding decyl analogue DB or for Q_2.

Partition of Ubiquinone Homologues in Phospholipid Bilayers. The partition coefficients determined from fluorescence quenching (Table I) have been compared with those determined from water solubility (eq 2).

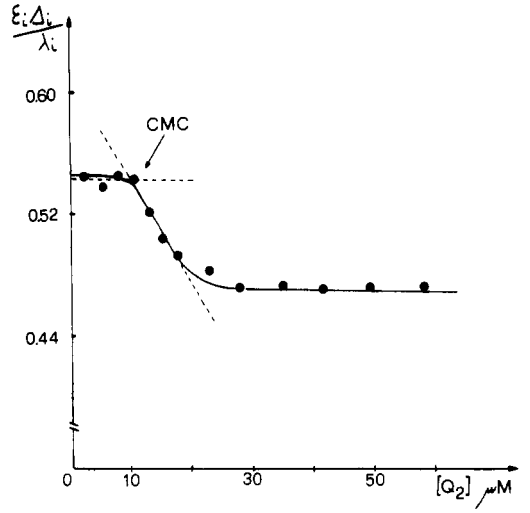

FIGURE 2: Effect of Q_2 concentration in aqueous medium on the parameter $\epsilon_i\Delta_i/\lambda_i$ obtained from spectrophotometric determinations. The above parameter is proportional to the transition dipole moment μ_i according to the equation (Kasha, 1963) $\mu_i^2 = a(\epsilon_i\Delta_i)/\lambda_i$, where ϵ_i is the millimolar extinction coefficient at the absorption maximum i, λ_i is the wavelength of the absorption maximum in nanometers, Δ_i is the half-bandwidth at λ_i, and a is a constant. The first inflection in the plot was taken as the critical micelle concentration, where micelles start to appear. The second inflection depends on the large increase of the ratio of micelles/monomers, so that the value of $\epsilon_i\Delta_i/\lambda_i$ for micelles becomes prevalent (M. Battino and G. Lenaz, unpublished results).

Table II: Critical Micelle Concentration and Partition Coefficients of Short Isoprenoid Chain Ubiquinone Homologues[a]

ubiquinone	cmc (μM)	solubility (mol/mol of H_2O × 10^8)	ΔG_{mic} (kcal/mol)	partition coeff [(mol of Q/mol of PL)/(mol of Q/mol of water) × 10^{-4}]
Q_2	14.0	25	−9.0	800
Q_3	0.9	1.6	−10.6	12500
ubiquinol 2	40	72	−8.3	280
ubiquinol 3	3.3	5.9	−9.8	3380

[a] Values determined experimentally in potassium phosphate buffer, 25 mM, pH 7.4, containing 1 mM EDTA, at 25 °C.

The solubility of Q_2 and Q_3 was determined by calculating the critical micelle concentration of the quinones. For such homologues it is possible to follow the spectral changes due to aggregation in water by monitoring their spectral parameters as a function of concentration. The UV band progressively shifts its maximum from 278 nm (indicative for a monomeric dispersion in a polar medium) to ≥282 nm, with a concomitant decrease of the apparent extinction coefficient (Degli Esposti et al., 1981a,b; Lenaz & Degli Esposti, 1985). An apparent cmc can be extrapolated from plots of λ_{max} or absorbance as a function of Q concentration (Figure 2). From the plots, the cmc in the medium used for fluorescence quenching were found to be 14 μM for Q_2 and 0.6 μM for Q_3 (Table II). With eq 2, partition coefficients were derived and plotted as a function of the isoprenoid chain length [cf. Figure 2 in Fato et al. (1985)]; the same figure also reports the P value obtained from fluorescence quenching; apart from Q_1, deviations are observed for the higher Q homologues.

The free energy of partition

$$\Delta G = -RT \ln P \qquad (6)$$

has been plotted as a function of isoprenoid chain length; the intercept on the ordinate axis gives the free energy of partition of the quinone ring in a hydrophobic phase. For the oxidized quinone ring a value of −4.5 kcal/mol was found, identical with that obtained by the partition coefficient of Q_0 (Ragan, 1978), indicating that the free energy required to remove the quinone ring from the hydrophobic phase into water is rather high.

Table III: Quenching Constants and Partition Coefficients of Different Q Homologues in Asolectin Vesicles Enriched with Cholesterol As Determined by Fluorescence Quenching of 12-AS

	−cholesterol		+cholesterol	
ubiquinone	$k_m \times 10^{-9}$ (M^{-1} s^{-1})	$P \times 10^{-4}$	$k_m \times 10^{-9}$ (M^{-1} s^{-1})	$P \times 10^{-4}$
Q_1	3.3	9.3	2.7	2.9
Q_3	3.6	18.5	2.2	
Q_7 (cosonicated)	3.9	3.2	1.5	0.6
16-NS	1.1	25.4	1.1	10.5

Table IV: Quenching Constants and Partition Coefficients of Q Homologues in Phospholipid Vesicles Using Different Fluorescent Probes

ubiquinone	probe	$k_m \times 10^{-9}$ (M^{-1} s^{-1})	$P \times 10^{-4}$	polarization
Q_3	3-AS	5.2	25.0	0.14
	6-AS	6.4	13.0	0.14
	12-AS	7.6	9.0	0.08
	DPH	15.2	5.7	
	ANS	7.9	6.7	
16-NS	DPH	1.0	4.8	
Q_0	DPH	11.2	0.23	
Q_1	DPH	5.0	2.7	

Under the experimental conditions for the quenching studies, using phospholipid concentrations of 0.5–4 mg/mL [i.e., $\alpha_m = (0.5–4) \times 10^{-3}$], the percentage of ubiquinones present in the water phase has been calculated by the equation (Ragan, 1978)

$$\text{mol of } Q_w = \frac{\text{mol of } Q_T}{(0.018P)(\text{mol of PL}) + 1} \qquad (7)$$

where Q_w is quinone in the water phase, Q_T is the total quinone present, P is the partition coefficient, and PL are phospholipids. With this equation it is calculated that Q in the water phase (or anyway unavailable to the fluorescent probe) in the quenching experiments usually ranges between 2% and 15%.

Factors Affecting Fluorescence Quenching by Ubiquinone Homologues. Cholesterol exerts a well-known rigidizing effect on fluid phospholipid bilayers [cf. Lenaz (1984)]; the effect of cholesterol on the quenching of 12-AS fluorescence by different ubiquinones has been tested, and the results are shown in Table III; it is obvious that cholesterol incorporation decreases both the partition coefficients of ubiquinones and the quenching constants k_m, in line with its negative effect on lipid fluidity. On the other hand, increase of viscosity of the external medium, obtained by increasing sucrose concentrations, had no effect on the quenching constants.

Extensive evidence is now available that the 9-anthroyloxy moiety of the AS probes is located in the membrane at depths corresponding to the position of the fluorophore in the fatty acid (Podo & Blasie, 1977; Haigh et al., 1979; Thulborn, 1981; Vincent et al., 1982; Chalpin & Kleinfeld, 1983; Chatelier & Sawyer, 1985), DPH is freely located in the hydrocarbon region (Shinitzky & Barenholz, 1978; Thulborn, 1981), and ANS is sitting in the glycerol region of the bilayer (Podo & Blasie, 1977). The apparent partition coefficients and the bimolecular quenching constants for Q_3 are listed in Table IV, also showing the fluorescence polarization of the probes in the absence of added quinones. The decreased polarization of the probes extending at segments deeper than C-6 agrees with the fluidity increase demonstrated with different physical techniques (Seelig, 1977; Gaffney & McNamee, 1974; Quinn,

Table V: Lateral Diffusion Coefficients of Different Ubiquinones in Asolectin Vesicles at 25 °C

ubiquinone	$D \times 10^6$ ($cm^2\ s^{-1}$)[a]	ubiquinone	$D \times 10^6$ ($cm^2\ s^{-1}$)[a]
Q_1 added	5.1	Q_{10} cosonicated (R = 24)[b]	1.0
Q_2 added	9.6	Q_{10} cosonicated (R = 19)[b]	1.3
Q_3 added	5.8	Q_{10} cosonicated (R = 40)[b]	0.5
Q_7 cosonicated	6.1	DB added	9.4
Q_{10} cosonicated (R = 8)[b]	3.5	NBQ added	7.8
		SBQ added	3.0
Q_{10} cosonicated (R = 14)[b]	1.9		

[a] After subtraction of the D_p values determined for 12-AS (cf. text for experimental details). From the k_m (Table I), the value of D_p from eq 5 was 0.25×10^{-6} $cm^2\ s^{-1}$. [b] The value of R to feed into eq 5 was taken as 4 Å for the probe and 4, 10, 20, 15, and 36 Å, respectively, for Q_{10}, according to different possible situations described in Figure 5.

1981). The results in Table IV show a progressive decrease of the apparent partition coefficient for Q_3 from the surface to the core of the bilayer, accompanied by an increase of the quenching constant. The apparent partition coefficient calculated from ANS fluorescence quenching was the highest found for Q_3. It is to be noted that the partition and quenching constants only vary within 1 order of magnitude with any of the probes tested.

The effect of temperature was evaluated on the quenching of 12-AS by Q homologues; from Arrhenius plots, an activation energy of 1.5–2 kcal/mol was calculated, a low value agreeing with a diffusional mechanism. In dimyristoyllecithin (DML) multilamellar bilayers, a temperature increase enhanced both P and k_m; at temperatures below the phase transition of DML (23.9 °C), the quenching is independent of PL concentration, suggesting the occurrence of phase separation.

Calculation of Diffusion Coefficients. Lateral diffusion coefficients calculated for the different Q homologues by the Smoluchowski equation are reported in Table V. The values obtained are strongly dependent on the molecular radii assumed for the quinones; by taking values ranging from 8 to 40 Å (see Discussion) for the sum of probe *plus* quinone, D would decrease by a factor of 7.

From the Smoluchowski equation, the sum of D_p plus D_q is readily obtained. The diffusion coefficient of the probe 12-AS was calculated from the quenching constant of 12-AS with a spin probe derivative of stearic acid (Table I). The quenching constant with 16-NS is about 3-fold higher than that with 5-NS; since the *lateral* diffusion of the two molecules is likely to be very similar, it is suggested that the quenching occurs by the high tumbling frequency of the former nitroxide at the tip of the fatty acyl chain in a cone including the bulkier anthroyloxy probe [cf. Blatt & Sawyer (1985) for results identical with those reported here and for discussion]. Since the quenching constants by 5-NS of the AS derivatives having the fluorophore at different positions in the chain are almost identical (Blatt & Sawyer, 1985), it is likely that 5-NS is a more faithful reporter of lateral diffusion than 16-NS. Since AS and NS molecules occupy similar positions in the membrane, the $D_p + D_q$ obtained by the Smoluchowski equation have been divided by 2, yielding a D value of 2.5×10^{-7} $cm^2\ s^{-1}$ for either 12-AS or 5-NS. This value was subtracted from the $D_p + D_q$ obtained by quenching, giving D_q for the different quinones. The diffusion coefficients for the ubiquinone series (Table V) range between 3×10^{-6} $cm^2\ s^{-1}$ and 9×10^{-6} $cm^2\ s^{-1}$, if we assume a quinone radius of 4 Å and a quenching efficiency of 1. Similar calculations assuming larger radii are reported for Q_{10} in the same table. Assuming the Q_{10} head group sweeping the membrane thickness, the molecule can be depicted as a disk of 40 × 30 Å (cf. Discussion). Application of the Smoluchowski equation to the experimental k_m yields $D = 1.3 \times 10^{-6}$ $cm^2\ s^{-1}$. This lower value appears to be the most acceptable one.

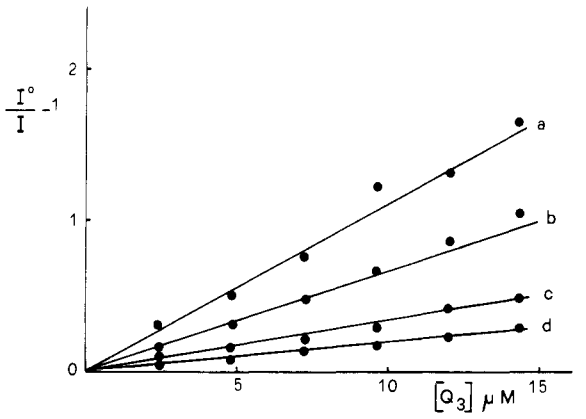

FIGURE 3: Stern–Volmer plots of quenching of 12-AS fluorescence by Q_3 at 30 °C added to EDTA–particles at different membrane fractional volumes (α_m): (a) 0.25×10^{-3}; (b) 0.5×10^{-3}; (c) 1×10^{-3}; (d) 2×10^{-3}. The ratio of probe to phospholipids was 1:150. The straight line derived from the replot of $1/k_{app}$ vs. α_m yielded a k_m of 3.2×10^9 $M^{-1}\ s^{-1}$, with a correlation coefficient of 0.96.

The Smoluchowski equation applies for diffusion proceeding in tridimensional paths; although the movement of a small molecule in a membrane may well be in three dimensions (Lakowicz & Hogen, 1980), a molecule having a fixed orientation in the membrane, like a polypeptide, would move only in two dimensions. It is not unlikely that ubiquinone molecules are obliged in membranes to follow substantially bidimensional paths. In such an eventuality we have applied to Q diffusion the treatment of Hardt (1979) for diffusion in two dimensions. At the quinone concentrations considered, the values calculated in the two ways (three vs. two dimensions) are in good agreement. Since the Hardt equation has been severely criticized (McCloskey & Poo, 1984), the Smoluchowski treatment for three-dimensional diffusion appears still sufficient to roughly describe our diffusion data.

Quenching of 12-AS Fluorescence by Ubiquinones in Mitochondrial Particles. We have applied the same technique to investigate the diffusion coefficients of ubiquinones in mitochondrial membranes. For practical reasons, since it was not feasible to incorporate Q homologues by cosonication, we have only investigated the fluorescence quenching of 12-AS by short isoprenoid homologues by direct addition to the assay medium (cf. Figure 3). The bimolecular constant k_m for quenching of 12-AS by Q_3 was found to be 3.2×10^9 $M^{-1}\ s^{-1}$ and the partition coefficient 2×10^5 on a mole fractional basis. These values were not affected by varying the probe concentration over a wide range. It may be observed that the diffusion coefficient of 4.7×10^{-6} $cm^2\ s^{-1}$, calculated by the Smoluchowski equation, compares well with the value obtained in phospholipid vesicles and reported in Table V. From temperature-dependence studies, the activation energy for quenching was found close to 1 kcal/mol, in good agreement with that in lipid vesicles.

EPR Studies of the Fluidity of Lipid Vesicles and Mitochondrial Membranes. The EPR spectra of 16-NS in phospholipid vesicles consist of three lines only, indicating that the segmental motion can be considered effectively isotropic. The pseudoisotropic rotational correlation time τ_c calculated from the spectra at 25 °C was on the average 0.8 ns; assuming a rotational radius for the spin-label $R = 4 \times 10^{-8}$ cm, η was calculated to be 0.12 P.

Similar experiments in different types of mitochondrial membranes, including EDTA-particles, yielded τ_c of about 1.2 ns at 25 °C corresponding to $\eta = 0.18$ P.

Kinetics of Ubiquinol–Cytochrome c Reductase and NADH:Ubiquinone Reductase Incorporated in Lipid Vesicles. The steady-state kinetics of ubiquinol–cytochrome *c* reductase were investigated at saturating cytochrome *c* concentrations; under these conditions the Lineweaver–Burk plots are monophasic (Degli Esposti & Lenaz, 1982a,b). With ubiquinol 1 as the electron donor, the isolated cytochrome bc_1 complex without addition of exogenous phospholipids and with 0.5 mg of endogenous phospholipid/mg of protein had a K_m for ubiquinol 1 of 37 μM; incorporation into lipid vesicles using three different levels of phospholipids did not change the K_m. The maximal turnover of the enzyme was 82 s^{-1} at the lower phospholipid addition, but increased to ca. 200 s^{-1} at the highest phospholipid concentration.

The phospholipid/water partition coefficient P of ubiquinol 1 is ca. 10^3 (in units of moles per liter). A double reciprocal plot of enzyme activity vs. ubiquinol 1 concentration *in the phospholipids*, calculated according to Ragan (1978), yields a K_m at all phospholipid concentrations of 35 mM *in the phospholipids*. The K_m in our experiments is therefore a value dependent upon quinone *concentration* in the lipid phase and not, as it is often mistakingly assumed, upon the quinone/enzyme ratio. Nevertheless, the maximal turnovers depended on the phospholipid concentration; a likely explanation is that phospholipids, as well as detergents, are necessary to maintain the complex in the monomeric active form.

A crude calculation (Rawn, 1983) allows determination of the second-order rate constant k_1 for the interaction of ubiquinol 1 with the bc_1 complex by using the equation

$$k_1 = k_{cat}/K_m \quad (8a)$$

where k_{cat} is the catalytic constant of the enzyme in s^{-1}. The k_{cat} for the bc_1 complex using ubiquinol 1 as the substrate was 200 s^{-1}; therefore

$$k_1 = 2 \times 10^2/3.5 \times 10^{-2} = 5.7 \times 10^3\ M^{-1}\ s^{-1} \quad (8b)$$

The K_m values of the bc_1 complex for ubiquinol 1 have been found to be dependent on a number of conditions (Degli Esposti & Lenaz, 1982a,b); however, even if the lowest value found is used (8 μM, corresponding to 8 mM in the phospholipids), the k_1 remains at the relatively low value of $2.5 \times 10^4\ M^{-1}\ s^{-1}$.

A similar calculation has been applied for the physiological ubiquinol 10 in mitochondrial membranes, using constants from the literature; with k_{cat} as high as 5000 s^{-1} (Zhu et al., 1982; Lenaz et al., 1985b) and an average K_m of 0.23 mol/mol of complex (Zhu et al., 1982; Poore & Ragan, 1982) (equivalent to 1.15 nmol/mg of protein in mitochondria, or roughly 2.3 mM in the phospholipids), we can again calculate

$$k_1 = 5 \times 10^3/2.3 \times 10^{-3} = 2.1 \times 10^6\ M^{-1}\ s^{-1} \quad (8c)$$

These values approach those of the second-order rate constants directly measured by pre-steady-state kinetic studies; the k_1 determined by us by direct stopped-flow experiments [cf. Lenaz et al. (1985a)] was $4 \times 10^4\ M^{-1}\ s^{-1}$ using the isolated complex and ubiquinol 1 as the substrate, in agreement with the value of $4 \times 10^4\ M^{-1}\ s^{-1}$ for the bc_1 complex from yeast mitochondria [Kauten, 1985; note also the value of $(1–2) \times 10^5\ M^{-1}\ s^{-1}$ obtained by Snozzi and Crofts (1984) for endogenous Q_{10} in phospholipid-enriched *R. sphaeroides* chromatophores].

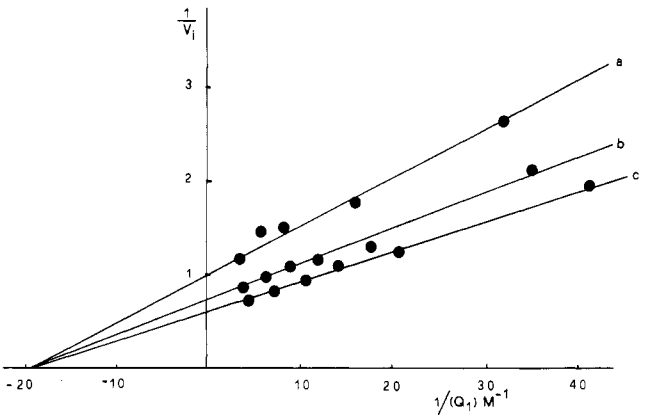

FIGURE 4: Lineweaver–Burk plots of NADH:ubiquinone 1 reductase in isolated complex I reconstituted with different phospholipid concentrations, using ubiquinone 1 as the variable substrate. The concentration of NADH was 0.1 mM and that of complex I was 5.4 μg/mL. The values of V_i are expressed in micromoles of NADH oxidized per minute per milligram of protein. Phospholipid concentrations were (a) 0.5 mg/mg of protein (endogenous phospholipids), (b) 5 mg/mg of protein, and (c) 15 mg/mg of protein. The data on the abscissa are expressed as ubiquinone 1 concentrations in the phospholipids (see text for explanation).

Similar experiments and calculations have been performed for ubiquinone reduction by NADH, catalyzed by the NADH:Q reductase complex; from double reciprocal plots at different phospholipid concentration, the apparent K_m for ubiquinone 1 ranged from 13.9 to 23.2 μM, in agreement with the data of Cabrini et al. (1981) and Ragan (1978, 1985); the K_m increased at increasing phospholipid concentration, a difference from what was found for the bc_1 complex. By use of the partition coefficient of 2.2×10^3 (in moles per liter), a K_m of 52.6 mM in the phospholipids was obtained at all phospholipid concentrations (cf. Figure 4). The difference with the bc_1 complex for the plots using the quinone concentrations in the total medium is due to the higher phospholipid concentrations present in the assays in the experiments with complex I, which has a lower activity compared with complex III.

Contrary to the results of Ragan (1978), the velocity is hyperbolically related to ubiquinone concentration in the phospholipids and not to the total amount of ubiquinone partitioned in the phospholipids. The reason for the discrepancy may lie in the different method of incorporation of the complex in the phospholipid vesicles; in fact, the addition of phospholipids to the diluted complex I in buffer may have prevented a homogeneous distribution of the enzymic units in the lipid vesicles, whereas mixing phospholipids and enzyme in cholate *before* the enzymatic assay ensures a complete homogenization of the enzyme in the phospholipid vesicles.

From the value of the k_{cat} of NADH:Q_1 reductase [370 s^{-1}, the same as obtained by Ragan (1985)], we can calculate

$$k_1 = 3.7 \times 10^2/5.26 \times 10^{-2} = 7 \times 10^3\ M^{-1}\ s^{-1} \quad (8d)$$

For evaluating the second-order rate constant for Q_{10} and complex I, we have used data from Kröger et al. (1973a) in uncoupled submitochondrial particles; with a V_{max} for NADH oxidation of 2.7 μmol·min^{-1}·mg^{-1} and a content of complex I of 0.065 nmol/mg of protein (Ragan, 1985), a turnover number of 700 s^{-1} is calculated for NaDH:Q reductase. The K_m for Q_{10} in NADH:Q reductase has been extrapolated from the saturation of reactivation of NADH oxidase by Q_{10} (Kröger et al., 1973a) and found to be 16 nmol/mg of protein, or 32 mM in the mitochondrial phospholipids. Calculation of k_1 as above yields

$$k_1 = 7 \times 10^2/3.2 \times 10^{-2} = 2.2 \times 10^4\ M^{-1}\ s^{-1} \quad (8e)$$

Discussion

Partition of Ubiquinone Homologues in Lipid Bilayers and Mitochondria. As experimentally determined, the partition of Q homologues in the lipid bilayers reflects the probability of the monomeric form added from ethanolic solution entering the membrane or forming an aggregate in water (Degli Esposti et al., 1981a):

$$Q_{int(monomer)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Q_{ext(monomer)} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Q_{ext(aggregate)}$$

where $P_{obsd} \approx k_1/k_2$ if $k_1 \gg k_{-1}$ and $k_2 \gg k_{-2}$.

The partition coefficient of Q_1 in phospholipid bilayers, calculated from the quenching measurements (cf. Table I), is in good agreement with that reported by Ragan (1978) by either a kinetic approach or from solubility and by Kingsley and Feigenson (1981) by direct determination after centrifugation. It is, however, higher than that measured by Degli Esposti et al. (1981a) by incorporation and spectrophotometric analysis after pentane washing of the unbound quinone, probably reflecting loss of the quinone by the pentane wash, as a consequence of the low values of k_1 and k_2 compared with k_{-1}. On the contrary, the coefficients for the Q homologues having chains of two isoprenoid units or more are lower when determined by fluorescence quenching than those expected from solubility and those reported by Ragan (1978), but agree with those determined by Degli Esposti et al. (1981a) and Stidham et al. (1984). The discrepancy reflects the fact that part of the Q added to the system exists in a form that is not accessible for quenching, either aggregating in water or adhering to the lipid bilayers, or nonmonomeric forms in the bilayer, which may have a lower quenching efficiency.

The values found for Q_7 and Q_{10} after cosonication with the phospholipids by quenching measurements are higher than those found after simple incubation but still far from the theoretical values. Sonication would force the mixing of the Q molecules with the phospholipid molecules in the same bilayers, probably increasing both k_1 and k_{-2}.

It is likely that the Q that does not partition in the lipids is present as an aggregated form in the aqueous phase (Stidham et al., 1984); nevertheless, part of the Q molecules might be present also in the bilayer as nonmonomeric forms having a lower quenching efficiency.

The apparent partition coefficients of Q_3 were affected by the localization of the fluorescent probes in the membranes and were slightly higher when calculated with superficial probes (ANS, 3-AS). Such results agree with a large distribution of the Q_3 molecules near the membrane surface, as also suggested by other studies (Kingsley & Feigenson, 1981; Stidham et al., 1984; Ulrich et al., 1985). Chatelier and Sawyer (1985), using a set of anthroyloxy fatty acid derivatives, found some evidence for two pools of ubiquinone 10 in the transverse membrane plane in mitochondria, one near the surface and the other localized in the midplane. The interchange between the two compartments, if any, must be slower than the nanosecond time scale on which the fluorescence quenching occurs. Transmembrane rates for ubiquinones were measured by Kingsley and Feigenson (1981) by proton NMR using lanthanide shift reagents and were found to range between 20 and 300 s^{-1}, depending on the homologue considered; with these rates, hardly any movement would be detected on the time scale of the fluorescence decay. By studies of the accessibility of ubiquinones to sodium borohydride and of the methoxy proton resonances in phospholipid bilayers, Ulrich et al. (1985) also concluded that Q_{10} is localized in two pools of comparable size, a superficial one and a deep one [cf. also Alonso et al. (1981) and Katsikas & Quinn (1982a,b, 1983)].

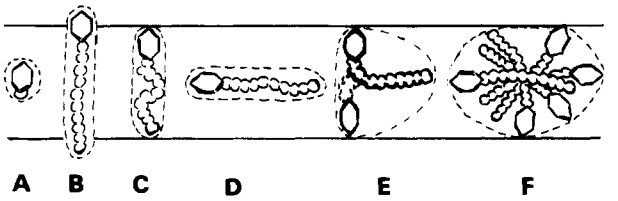

FIGURE 5: Possible dispositions of Q molecules in phospholipid bilayers: (A) Q_1 ($R \approx 4$ Å); (B) Q_{10} as an extended rod perpendicular to the bilayer plane ($h \approx 50$ Å; $a \approx 4$ Å); (C) Q_{10} as a rod perpendicular to the bilayer plane but not protruding out of the membrane ($h \approx 40$ Å; $a \approx 4$ Å); (D) extended rod parallel to the bilayer plane ($h \approx 8$ Å; $a \approx 20$ Å); (E) bent molecule, with the quinone ring spending most of its time near the polar heads of the lipids ($h \approx 40$ Å; $a \approx 30$ Å); (F) micellar model with several Q molecules forming an ellipsoid ($h \approx 40$ Å; $a \approx 40$ Å).

The present study on partition and determination of cmc for the Q homologues in aqueous medium shows that the free energy for transfer of the quinone ring from water to a hydrophobic medium is rather negative, allowing its significant localization in the hydrophobic portion of the membrane. From the micellization properties of mixed lecithin–Q dispersions in ethanol–water (Lenaz & Degli Esposti, 1985), the cmc appeared largely independent of the quinone hydrophobicity, contrary to pure Q micelles in the same system. The results suggest that, if the ubiquinone molecules stack between the phospholipid molecules, they do so by keeping the quinone ring in a nonpolar phase, and the critical length of the isoprenoid chain for stacking is 3–4 units, the remainder being accommodated in such a way that it does not contribute to the net thermodynamics of the system. It may be inferred from these results that ubiquinone may be realistically considered as switching the position of its quinone ring from near the membrane surface (but still in a nonpolar environment) to the hydrophobic core, while the hydrophobic tail is largely accommodated in the hydrophobic interior in the midplane of the membrane.

Diffusion Coefficients of Ubiquinones. The ubiquinone homologues from Q_1 to Q_{10} in their oxidized forms have high diffusion coefficients ranging from 10^{-6} to 10^{-5} $cm^2\ s^{-1}$, as measured from the bimolecular collision constant determined by the quenching of fluorescence of probes located in lipid bilayers (Table V). The validity of the method has been assessed by a number of critical tests, and it was found to be reliable as discussed under Results. In particular, we have eliminated the possibility that high quenching constants are the result of proximity effects of ubiquinones and probes within aggregates or clusters either in water or within the membrane phase. Values of the diffusion coefficients in the same range have been found in submitochondrial particles and in mitochondria.

The diffusion coefficient of the spin-labeled fatty acid 5-NS was found by the same method to be 2.5×10^{-7} $cm^2\ s^{-1}$; this value is of the same order as those calculated by Stier and Sackmann (1973) from the concentration dependence of the spin-exchange frequency in liver microsomes (1×10^{-7} $cm^2\ s^{-1}$) and those found by Galla and Sackmann (1974) and Galla et al. (1979) by excimer formation of pyrene fatty acid derivatives.

The diffusion coefficients for the Q series depend on the collisional radii of quinone and probe, which on their hand would depend on their localization in the membrane. Ignorance of the localization and orientation of Q_{10} in the bilayer can yield an uncertainty factor of almost 1 order of magnitude,

if we assume a minimal collisional radius of 4 Å for the quinone extending as a stiff rod perpendicular to the plane of the membrane and a maximal radius of 40 Å for Q clustered in an ellipsoid micelle [cf. Quinn & Katsikas (1985)] (Figure 5). We exclude the micellar model, in accordance with the physical studies of Stidham et al. (1984) and on the basis of the fact that the quenching constants for Q_{10} are not far different from those of the short Q homologues, which would hardly form micelles. We also exclude the former situation, since the elongated Q_{10} molecule is too long to be accommodated parallel to the lipid molecules. Moreover, calculation of average bilayer viscosity using the equation of Saffman and Delbrück (1975) for viscous drag of the outer medium and our experimental diffusion coefficient would yield a viscosity too low for a lipid bilayer including the polar heads of the phospholipids (Hughes et al., 1982; Shinitzky & Inbar, 1976). The conclusion is that Q_{10} moves in a fluid medium not in contact with the aqueous phase and the polar heads of the phospholipids. Since for motion at the membrane interior the equation of Saffman and Delbrück is no more applicable (Vaz et al., 1984), we have used calculations based on random walk in homogeneous medium in two dimensions (Berg, 1983). In such a case the viscosity in the midplane can be calculated to be 10 cP or less, not far from the experimental value obtained by the rotational correlation time of the 16-NS spin-label and the values accepted in the literature (Seelig & Seelig, 1980; Dix et al., 1978; Brown et al., 1979; Small et al., 1984).

Two possibilities appear likely for the localization of Q_{10} in the membrane: either Q fully extended in the membrane midplane [cf. Crane (1977)] or a mixed orientation averaging a spherical molecule. We propose that Q_{10} laterally diffuses in the bilayer in two dimensions, but with the quinone ring oscillating between the two bilayer surfaces, keeping in a hydrophobic environment not extending beyond the glycerol region. This localization is compatible with the studies discussed in the previous section. Assuming the Q_{10} head group sweeping the membrane thickness, the molecule can be depicted as a disk of 40 × 30 Å; application of the Smoluchowski equation to the experimental quenching constant yields $D = 1.3 \times 10^{-6}$ cm^2 s^{-1} (cf. Table V).

In mitochondrial membranes, the same conclusions can be reached. The inner mitochondrial membrane is very fluid (Schneider et al., 1982), and the reported high viscosity (Shinitzky & Inbar, 1976) may be overestimated by the fluorescence polarization technique (Jähnig, 1979; Meier et al., 1982). From the rotational correlation time of the spin probe 16-NS we have calculated a viscosity of 0.18 P (in comparison with 0.12 P for asolectin vesicles). Accordingly, the lateral diffusion coefficient of Q_3 in mitochondrial membranes is only slightly lower than in lipid vesicles.

The diffusion coefficients obtained for ubiquinone in this work are about 3 orders of magnitude greater than those found by Gupte et al. (1984) using the method of FRAP on a fluorescent decylubiquinone derivative, although these authors have calculated that even such low values would not limit electron transfer under physiological conditions. This discrepancy cannot be solely explained by the position of the quinone derivative used by Gupte et al. This quinone was assigned to the polar glycerol region of the bilayer on the basis of its spectroscopic properties, in contrast with the quinones used in this investigation, whose UV spectra indicate that they are located in a nonpolar region of the membrane (Degli Esposti et al., 1981a,b). It has been demonstrated that diffusion coefficients calculated for nonplanar membrane surfaces are underestimated at least by a factor of 2 (Aizenbud & Gershon, 1982), since they represent mobility on projected flat planes of real nonflat membranes. Mitochondrial cristae are highly convoluted membrane surfaces, although the swollen mitochondria used by Gupte et al. should reduce this underestimation to a minimum.

An additional reason for the discrepancy may lie in the fact that FRAP measures long-range diffusion, and the presence of integral membrane proteins might increase the total path length for quinone diffusion over large areas. A superfast collisional rate may find an explanation in a nonrandom distribution of the reactants (Nachliel & Gutman, 1984), since the Smoluchowski equation for diffusion-controlled second-order reactions assumes that the reactants are randomly dispersed in a very large volume. In mitochondrial membranes where proteins are highly packed and microheterogeneity in the plane of the membrane is likely to exist (Jain, 1983), facilitated collisions are likely to take place. Such facilitated paths would lead to overestimation of the measured diffusion coefficients (Kell, 1983). This explanation is unlikely to hold for the lipid bilayers above the transition temperature, where quinone and probe molecules are probably randomly dispersed. There may be another reason, however, for a "short-range" diffusion coefficient [cf. Berg & Von Hippel (1985)], if the quenching is affected by encounters between Q's fixed, for the time of interest, in the plane of the membrane but free to sweep out a cone including the fluorescent reporter.

The contribution of the "flipping" motions of the quinone ring in the collisional encounters of long-chain quinones with the probe is likely, as demonstrated by the higher quenching by 16-NS than by 5-NS, in accordance with a high mobility of the probe free to tumble at the tip of the acyl chain (Blatt & Sawyer, 1985); this motion, however, should not contribute more than by a factor of 3 over the true *lateral* displacement of the quinones. In the case of the shorter chain ubiquinone homologues, which also have high quenching constants, such flipping motion is obviously not possible. Since the contribution of short-range encounters should not be above 1 order of magnitude, the discrepancy between the diffusion coefficients found in this study ($\geq 10^{-7}$ cm^2 s^{-1}) and those found by Gupte et al. (1984) ($\leq 10^{-9}$ cm^2 s^{-1}) is still largely unexplained.

The physiological relevance of long-range and short-range diffusion could be different, depending on the model assumed for the organization of the respiratory chain [cf. Rich (1984)]; for a random distribution of redox complexes and ubiquinone, long-range diffusion might be predominant, whereas in a solid-state assembly of the chain, with rapid association–dissociation equilibrium of the quinone, short-range diffusion would become predominant.

Our method cannot provide direct values for diffusion coefficients of reduced ubiquinones; however, even if their higher polarity could change their location in the membranes (Spisni et al., 1978; Degli Esposti et al., 1981a), there is little reason to believe that this fact would grossly affect the diffusional rates.

Our model for Q diffusion in mitochondria is well suited for its bioenergetic role, since the sites in the enzymes that reduce or oxidize Q appear to be close to the membrane surfaces [e.g., the iron–sulfur cluster of complex III; cf. Wikström and Saraste (1984)] as also required by the Q-cycle model (Mitchell, 1976). Of course our data cannot describe the eventual properties of a small fraction of Q molecules bound to proteins in a stable association.

Ubiquinone Diffusion and Turnover of Ubiquinone-Requiring Enzymes. The large diffusion coefficients measured in this work for ubiquinone in lipid bilayers and mitochondrial

membranes confirm the kinetic results of Kröger et al. (1973a,b) that lateral diffusion of quinones is not rate limiting for electron transfer.

The lateral displacement rate d/t is calculated by the relation

$$d^2 = 4Dt \quad (9)$$

for a two-dimensional path, where d is the distance run by the diffusing molecule and t the time; if we assume that the average distance between complex I and complex III in the inner mitochondrial membrane is 30 nm (Capaldi, 1982), the distance would be covered in 0.0022 ms for a diffusion coefficient of 10^{-6} cm^2 s^{-1}. For diffusion of a particle to a small target of diameter r, the displacement d from a diffusion coefficient D is affected by the logarithm of r/d in two dimensions (Adam & Delbrück, 1968; Berg & Purcell, 1977):

$$t = (d^2/2D)[\ln (d/r) - 3/4] \quad (10)$$

In particular, a distance of 30 nm for ubiquinone moving from complex I to complex III, with a diameter of complex III monomer of 7 nm (Rieske & Ho, 1985) and a diffusion coefficient of 10^{-6} cm^2 s^{-1}, would be covered in 0.003 ms. The turnover of mitochondrial redox complexes may exceed 1000 s^{-1}, even if *lower* turnovers are expected for integrated electron transfer between complexes according to the mobile pool equation (eq 1); under normal conditions, the turnover of the respiratory chain should not be beyond 200 s^{-1} (5 ms/turnover), leaving a large excess for the lateral displacement rate. On the other hand, diffusion of the complexes [which should correspond to $D = 10^{-9}$–10^{-10} cm^2 s^{-1} (Gupte et al., 1984; Sowers & Hackenbrock, 1981)] seems clearly incompatible with the turnover of the respiratory chain.

Since collisional encounters, particularly in two dimensions, should be quite effective for electron transfer, it is to be expected that even a large dilution of the quinone pool in the mitochondrial membrane would not render the diffusion rate limiting. This prediction appears to be contradicted by the study of Schneider et al. (1982), who found that 2–5-fold dilutions of the ubiquinone pool in the inner mitochondrial membrane drastically reduce the rate of integrated electron transfer (NADH:cytochrome *c* reductase and succinate:cytochrome *c* reductase). The reason for such an effect, however, appears to be inherent in the kinetic features of the ubiquinone interactions with the partner enzyme complexes. In other words, the effect of dilution does not mean that Q *diffusion* has become rate limiting; in fact, the decrease of Q concentration below the K_m of its partner complexes for the quinone is sufficient to explain the loss of activity. The K_m values of 2.4 mM in the phospholipids of complex III for ubiquinol 10 and 32 mM in the phospholipids of complex I for ubiquione 10 (cf. Results) and the known turnovers of complexes III and I appear sufficient to explain a substantial decrease of NADH:cytochrome *c* reductase by dilution, by applying the pool equation (eq 1).

By a different kind of reasoning we have demonstrated that the interactions of Q with complex I and QH_2 with complex III are not diffusion limited, that is to say, no contribution of diffusion is given to k_1, the second-order rate constant of enzyme–substrate redox interaction. Comparison of the bimolecular collision constants obtained from the quenching experiments and the second-order rate constant k_1 for both enzymes shows a discrepancy by a factor of $\geq 10^3$. Thus, it is quite clear that diffusion will not be a limiting factor. Either the reorientation of the quinone in the active site or the binding and unbinding of the quinone, or, anyway, some catalytic steps in the reaction mechanism contributing to k_1 are therefore the rate-limiting steps in the computed or experimental second-order rate constants. The activation energy derived from Arrhenius plots of ubiquinol–cytochrome *c* reductase in the native membranes or in the bc_1 complex inlaid in phospholipids is 8–11 kcal/mol (Lenaz et al., 1985), well above the activation energy found for Q diffusion.

In conclusion, our study strongly supports the view that the concept of a homogeneous pool of ubiquinone is sound and that diffusion is not the rate-limiting reaction controlling electron transfer under most reported experimental conditions. Under conditions of very limited lipid content, redox enzymes do not follow a homogeneous pool behavior. As suggested by Gutman (1985), this condition could bring about a diffusion-limited reaction by inducing aggregation of enzymes and increasing enormously the membrane viscosity.

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Registry No. Q_1, 727-81-1; Q_2, 606-06-4; Q_3, 1173-76-8; Q_7, 303-95-7; Q_{10}, 303-98-0; DB, 55486-00-5; NBQ, 101630-84-6; SBQ, 101630-85-7; DML, 13699-48-4; NADH:ubiquinone 1 reductase, 9028-04-0; complex III, 9027-03-6; cholesterol, 57-88-5.

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Bound Adenosine 5′-Triphosphate Formation, Bound Adenosine 5′-Diphosphate and Inorganic Phosphate Retention, and Inorganic Phosphate Oxygen Exchange by Chloroplast Adenosinetriphosphatase in the Presence of Ca^{2+} or Mg^{2+} †

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ABSTRACT: When the heat-activated chloroplast F_1 ATPase hydrolyzes [^{3}H,γ-^{32}P]ATP, followed by the removal of medium ATP, ADP, and P_i, the enzyme has labeled ATP, ADP, and P_i bound to it in about equal amounts. The total of the bound [^{3}H]ADP and [^{3}H]ATP approaches 1 mol/mol of enzyme. Over a 30-min period, most of the bound [^{32}P]P_i falls off, and the bound [^{3}H]ATP is converted to bound [^{3}H]ADP. Enzyme with such remaining tightly bound ADP will form bound ATP from relatively high concentrations of medium P_i with either Mg^{2+} or Ca^{2+} present. The tightly bound ADP is thus at a site that retains a catalytic capacity for slow single-site ATP hydrolysis (or synthesis) and is likely the site that participates in cooperative rapid net ATP hydrolysis. During hydrolysis of 50 μM [^{3}H]ATP in the presence of either Mg^{2+} or Ca^{2+}, the enzyme has a steady-state level of about one bound [^{3}H]ADP per mole of enzyme. Because bound [^{3}H]ATP is also present, the [^{3}H]ADP is regarded as being present on two cooperating catalytic sites. The formation and levels of bound ATP, ADP, and P_i show that reversal of bound ATP hydrolysis can occur with either Ca^{2+} or Mg^{2+} present. They do not reveal why no phosphate oxygen exchange accompanies cleavage of low ATP concentrations with Ca^{2+} in contrast to Mg^{2+} with the heat-activated enzyme. Phosphate oxygen exchange does occur with either Mg^{2+} or Ca^{2+} present when low ATP concentrations are hydrolyzed with the octyl glucoside activated ATPase. Ligand binding properties of Ca^{2+} at the catalytic site rather than lack of reversible cleavage of bound ATP may underlie lack of oxygen exchange under some conditions.

The CF_1 ATPase[1] component of the chloroplast ATP synthase retains a tightly bound ADP that has an apparent regulatory function [see review by Shavit (1980)] and appears to be at the catalytic sites on the β subunits of the enzyme [see Feldman & Boyer (1985) and references cited therein]. When chloroplasts are exposed to ^{14}C-labeled ATP, the isolated CF_1 ATPase retains a bound labeled ADP (Magnusson & McCarty, 1976). An adquate understanding of the factors

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[1] Abbreviations: CF_1 ATPase and MF_1 ATPase, ATPase portion of ATP synthase from spinach chloroplasts and beef heart mitochondria, respectively; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; EDTA, ethylenediaminetetraacetic acid.

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