Lateral Diffusion as a Rate-limiting Step in Ubiquinone-mediated Mitochondrial Electron Transport*

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Data are presented which indicate that the diffusion-based collisions of ubiquinone with its redox partners in the mitochondrial inner membrane are a rate-limiting step for maximum (uncoupled) rates of succinate-linked electron transport. Data were obtained from experimental analysis of a comparison of the apparent activation energies of lateral diffusion rates, collision frequencies, and electron transport rates in native and protein-diluted (phospholipid-enriched) inner membranes. Diffusion coefficients for Complex III (ubiquinol:cytochrome c oxidoreductase) and ubiquinone redox components were determined as a function of temperature using fluorescence recovery after photobleaching, and collision frequencies of appropriate redox partners were subsequently calculated. The data reveal that 1) the apparent activation energies for both diffusion and electron transport were highest in the native inner membrane and decreased with decreasing protein density, 2) the apparent activation energy for the diffusion step of ubiquinone made up the most significant portion of the activation energy for the overall kinetic activity, i.e. electron transport steps plus the diffusion steps, 3) the apparent activation energies for both diffusion and electron transport decreased in a proportionate manner as the membrane protein density was decreased, and 4) Arrhenius plots of the ratio of experimental electron transport to productive collisions (turnovers) to calculated theoretically predicted, diffusion-based collisions for ubiquinone with its redox partners had little or no temperature dependence, indicating that as temperature increases, increases in electron transport rate are accounted for by the increases in diffusion-based collisions. These data support the Random Collision Model of mitochondrial electron transport in which the rates of diffusion and appropriate concentrations of redox components limit the maximum rates of electron transport in the inner membrane.

This laboratory has postulated a Random Collision Model of mitochondrial electron transport based on a large number of observations on the structure of the mitochondrial inner membrane, the kinetics of electron transfer, and the diffusion of redox components (1–13). The model envisions highly mobile, independent, redox components that specifically transfer reducing equivalents following one or more diffusion-based random collisions between them and predicts that rates of diffusion of the redox components at the appropriate concentrations are fundamental factors limiting the rate of mitochondrial electron transport.

Early studies (14, 15) have suggested that any rate-limiting step in the overall electron transport sequence should be found closer to the dehydrogenases than to the terminal oxidase, Complex IV (cytochrome oxidase). Consistent with the Random Collision Model, a likely candidate for a rate-limiting step would be the diffusion-based collisions of ubiquinone with its redox partners. Ubiquinone has long been thought to be a mobile carrier (16), having been shown to exist functionally as a homogeneous pool in the inner membrane bilayer (17, 18). Our laboratory has reported data indicating a ubiquinone diffusion-mediated electron transfer (19), has measured directly the diffusion rate of a ubiquinone analogue in the inner membrane (12), and has shown the multicollisional, obstructed, long-range diffusional nature of Q-dominated electron transport (13).

To determine a rate-limiting step in the complex sequence of consecutive reactions of mitochondrial electron transport, we have utilized the concepts put forth in the theory of rate processes (20, 21) that are derived in part from the theory of absolute reaction rates (22). The rate process approach involves the determination of temperature dependences of activation energies for both physical and/or chemical rate processes (e.g., diffusion and electron transfer). The E for a rate process reports on the potential energy barrier, i.e. the energy required to carry out a process. Based on rate process theory, it can be predicted that the E for the rate-limiting step in a series of consecutive reactions will be the most significant contribution to the E for the overall process. Thus, we have compared the temperature dependence of the overall diffusion steps to that of the overall electron transport steps in the reaction of ubiquinone with two of its redox partners, Complexes II (succinate:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase), in the inner membrane. Experimentally, this was accomplished by measuring the temperature dependences of the appropriate Ds determined by FRAP and measuring the temperature dependences of succinate-linked electron transport rates. The results from this analysis support diffusion-based collisions of ubiquinone with its redox partners.
partners as the rate-limiting step for overall mitochondrial electron transport in the inner membrane.

**Experimental Procedures**

**Membrane Preparations**—Liver mitochondria were isolated from male Sprague-Dawley rats according to published procedures using H$_{2}O$ isolation medium (300 mM isolation medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Heps, 0.5 mg/ml bovine serum albumin at pH 7.4) (23, 24). A controlled digitonin incubation (25) was used to selectively remove the outer membrane, leaving an intact inner membrane-matrix fraction, i.e. mitoplasts. The inner membrane-matrix was converted to a spherical shape while maintaining activity by washing in hypotonic H$_{2}O$ medium (40 mM medium made as a 7.5-fold dilution of H$_{2}O$ isolation medium without bovine serum albumin) (26).

The low pH method of Schneider et al. (23) was used to decrease the protein density of the spherical, native inner membranes by enrichment with exogenous phospholipid (asolecin) at a pH of 6.35 to yield four fractions of intact, functional inner membranes each with a different integral membrane protein density. Native (unenriched) or protein-diluted (phospholipid-enriched) inner membranes were used in kinetic measurements or fused on glass microscope slides to a 2-5-pm diameter sufficient for FRAP measurements (27).

**Fluorescent Probes and Membrane Labeling**—Fluorescent probes were utilized for the FRAP measurements of redox components. DiI was used to follow ubiquinone diffusion (2, 29), was conjugated with a tetramethylrhodamine isothiocyanate fluorophore (12), and used as an immunospecific label to follow the diffusion of Complex III in fused, ultralarge, native (unenriched) and protein-diluted (phospholipid-enriched) inner membranes or fused on glass microscope slides. Native (unenriched) and protein-diluted (phospholipid-enriched) inner membranes were used in kinetic measurements or fused on glass microscope slides to a 2.5-pm diameter sufficient for FRAP measurements (27).

**Comparisons of the Temperature Dependence of $Q_{c}^{	ext{NBDHA}}$**

| $T$ (°C) | $Q_{c}^{	ext{NBDHA}}$ | DiI |
|----------|-----------------|-----|
| 23.0     | 0.05            | 0.1 |
| 23.3     | 0.17            | 0.18|
| 23.6     | 0.4             | 0.4 |
| 23.7     | 1.5             | 1.5 |
| 24.0     | 2.9             | 3.0 |
| 25.0     | 3.1             | 3.2 |
| 26.0     | 4.0             | 3.8 |
| 27.0     | 4.1             | 4.2 |
| 28.0     | 4.3             | 4.5 |
| 29.0     | 4.6             | 4.9 |
| 30.0     | 4.9             | 5.0 |
| 31.0     | 4.9             | 5.0 |
| 32.0     | 5.0             | 5.1 |

**Lateral Diffusion in Mitochondrial Electron Transport**

Measurements, laser beam focusing, and membrane location; 2) ease and uniformity of membrane labeling; and 3) proven capability to report the $D$ for ubiquinones at different polar regions of the electron transport sequence required the prior determination of the temperature dependence of the $D$s for appropriate redox components. Therefore, $D$s of Complex III, as a model for integral membrane proteins, and DiI, as a model for ubiquinone (2, 29), were determined using FRAP in fused, ultralarge, native (unenriched) and protein-diluted (phospholipid-enriched) inner membranes at a given temperature using the signal-averaging capability of the FRAP instrument. Complex III and ubiquinone (DiI) measurements involved the signal averaging of no less than four and eight FRAP curves, respectively, for an individual membrane. At each temperature, 10–30+ inner membranes from different mitochondrial preparations were measured.

**Electron Transport Activities**—Maximum succinate oxidase activity was determined polarographically via a Clark-type oxygen electrode (36). The incubation medium consisted of 10 mM potassium phosphate, pH 7.4, 1 mM carbonyl cyanide m-chlorophenylhydrazone, and 13 μM cytochrome $c$ at a 5 mM sodium succinate was used to start the reaction. Maximum succinate:cytochrome $c$ oxidoreductase activity was determined using an SLM-Aminco DW2-C dual wavelength spectrophotometer to follow the reduction of cytochrome $c$ at the 550-540-nm wavelength pair. An extinction coefficient of 19.9 mM$^{-1}$ cm$^{-1}$ was used. The reaction medium consisted of 80 mM potassium phosphate, pH 7.4, 1 mM sodium succinate, 10 mM K$_{2}$H$_{2}$O$_{4}$, and 2 mM KCN; 50 μM cytochrome $c$ was used to start the reaction. Membranes were temperature-equilibrated for 1–2 min before beginning the reaction. For each temperature, 15–30 determinations were averaged. Temperatures were read directly in the thermostated oxygen electrode reaction chamber (oxidase assay) or spectrophotometer cuvette (oxidoreductase assay) using a Sensortek TS-2 or TS-4 microscope stage and controller. Determination of the actual sample temperature was accomplished by comparison to a calibration curve acquired using a surface thermocouple probe bonded to a glass slide to accurately correlate specimen temperature with the indicated temperature on the controller. FRAP curves were acquired from freshly fused, intact inner membranes at a given temperature using the signal-averaging capability of the FRAP instrument. Complex III and ubiquinone (DiI) measurements involved the signal averaging of no less than four and eight FRAP curves, respectively, for an individual membrane. At each temperature, 10–30+ inner membranes from different mitochondrial preparations were measured.

**Materials**—All reagents were reagent-grade. Cytochrome $c$ (Type VI from horse heart) was purchased from Sigma. Rhodamine isothiocyanate was obtained from Research Organics. DiI was obtained from Molecular Probes. Purified Complex III was the gift of Dr. Tsoo King (Institute of Structural and Functional Studies, Philadelphia, PA).

**RESULTS**

**Temperature Dependence of Lateral Diffusion Coefficients of Redox Components**—The determination of the $E_{a}$ for the diffusion step(s) in the ubiquinone region of the electron transport sequence required the prior determination of the temperature dependences of the $D$s for appropriate redox components. Therefore, $D$s of Complex III, as a model for integral membrane proteins, and DiI, as a model for ubiquinone (2, 29), were determined using FRAP in fused, ultralarge, native (unenriched) and protein-diluted (phospholipid-enriched) inner membranes at a given temperature using the signal-averaging capability of the FRAP instrument. Complex III and ubiquinone (DiI) measurements involved the signal averaging of no less than four and eight FRAP curves, respectively, for an individual membrane. At each temperature, 10–30+ inner membranes from different mitochondrial preparations were measured.

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riched), mitochondrial inner membranes over the 5–40 °C range.

Ds for both Complex III and ubiquinone (DiI) were found to increase as a function of increasing temperature for fused, native and protein-diluted, inner membranes. In each case, Arrhenius plots (log rate versus 1/T) of the Ds revealed single-phase, linear increases over the temperature range studied (Fig. 1). The temperature-dependent increase in the Ds was greatest in the native inner membrane for both Complex III (Fig. 1A) and ubiquinone (DiI) (Fig. 1B) as indicated by the $E_a$ calculated from the plots (Table II). Progressive decreases in membrane protein density resulted in increases in Ds (Fig. 1, C–F) with concomitant decreases in $E_a$ (Table II) for both Complex III and ubiquinone (DiI). Ubiquinone (DiI) showed greater Ds and $E_a$s than Complex III at each of the protein densities studied. Taken together, these data revealed substantial energy requirements for redox component diffusion in the inner membrane (see "Discussion") and indicated an important role for protein density in modulating the temperature dependence of the diffusion rates of membrane diffusants.

Temperature Dependence of Collision Frequencies of Redox Partners—The functional significance of the diffusion of redox components is realized in the frequency of the diffusion-based collisions of appropriate redox partners related to the frequency of electron transfer. Thus, we calculated the temperature dependence of the $D_s$ of the redox partners in terms of collision frequencies using the Hardt equation for two-dimensional diffusion (41), which quantifies the maximum possible (theoretical, diffusion-controlled) collisions of a pair of redox partners given their respective $D_s$, effective redox concentrations, and collision radius. We used this approach to calculate the collision frequencies for two pairs of redox partners, Complex II–Q and Q-Complex III, and at their effective redox concentrations, representing the two "half-reactions" of electron transport, at selected temperatures based on our experimental Ds and other parameters as detailed previously (12, 13). We would point out that with our rate process approach, any two-dimensional reaction diffusion equation may be used since such equations have no specific temperature-dependent term other than that implicit in the experimentally determined diffusion coefficients. Since the relationship as a function of temperature among individual points in the Arrhenius plot will not change with the equation used, the slope and therefore the apparent $E_a$ will be independent of the equation used.

Arrhenius plots of the temperature dependence of the collision frequencies for each pair of redox partners were monophasic and linear over the temperature range studied (e.g. Fig. 2, A and B). The greatest collision frequencies and the strongest temperature dependence were found in the native membrane for both Complex II–Q and Q-Complex III redox partners (Q-Complex III shown in Fig. 2A), compared to protein-diluted (II-Complex Q shown in Fig. 2B) inner membranes. The decrease in the temperature dependence of the redox partner collision frequency with decreasing protein density can be clearly seen in the decrease in the $E_a$s (Table III, second column). These results revealed a significant energy requirement for the diffusion-mediated, collisional interaction of Q with either of its redox partners at effective redox concentrations as affected by protein density, which, as expected, paralleled the energy requirement of the $D_s$.

Temperature Dependence of Diffusion Steps of Redox Partners—In order to relate the diffusion rate and collision fre-
frequency of redox components to the electron transport rate in the ubiquinone region, the temperature dependence of the diffusion of Q from Complex II to III, i.e. Q's respective subunits, the temperature dependence of the frequency of redox components to the electron transport rate in the electron transport rate in the combined

Complex II-Q and Q-Complex III half-reactions, we utilized Gutmann's treatment (42) of Kröger and Klingenberg's classic pool equation (17, 18) for ubiquinone-mediated electron transport, which provides a separation of the rate constants of the diffusion and electron transfer steps of the redox reactions. The diffusion term for Gutmann's equation is: $$V_{diff} = (V_r - V_l)/(V_r + V_l),$$ where $V$ is rate of the diffusion step, and subscripts $r$ and $l$ refer to the oxidation and reduction reactions, respectively (see also Equations 8 and 9 in Ref. 42). In adapting Gutmann's treatment for our analyses, we consider concepts derived from the theory of absolute reaction rates (22) and apply the theory of rate processes (20, 21). Substituting the appropriate rate process theory expression (20, 21) of the Arrhenius equation for our experimentally determined $E_a$ (Table III, second column) into the above equation, an $E_a$ (Table III, third column) for Q's electron-transporting diffusion step in the overall reaction of Q with both Complexes II and III is obtained. The data reveal that the $E_a$ for the diffusion step of Q with its redox partners is high and decreases with decreasing protein density (Table III, third column).

**Temperature Dependence of Electron Transport**—The temperature dependence of electron transport were determined to obtain $E_a$ for the appropriate overall kinetic electron transport reactions which contain both the electron transfer and diffusion steps. Succinate-linked activities were chosen since each contains the diffusion of Q from one of its reduc- tants, Complex II, to its only oxidant, Complex III. Maximum (uncoupled) succinate oxidase and succinate-cytochrome c oxidoreductase activities were determined in native (Fig. 3, A and B) and protein-diluted (Fig. 4, A and B) inner membranes.

**TABLE III**

| Membrane         | Redox sequence | Collision frequency | Diffusion step | Uncoupled e⁻ transport activity |
|------------------|----------------|---------------------|----------------|---------------------------------|
| Native           | II-Q           | 11.8                | 12.2           | 12.87 SCOR                      |
|                   | Q-III          | 11.9                | 12.2           | 14.3 SO                         |
|                   | II-Q-III       | 9.07                | 9.55           | 10.8 SCOR                       |
|                   | Q-III/7        | 9.2                 | 9.55           | 9.3 SO                          |
|                   | II-Q-III/7     | 8.6                 | 9.22           | 10.5 SCOR                       |
|                   | Q-III/7/14     | 8.9                 | 9.22           | 9.0 SO                          |

SCOR, succinate-cytochrome c oxidoreductase; SO, succinate oxidase.

Protein dilution by phospholipid enrichment (see "Experimental Procedures").
port processes (maximum, succinate-linked activities) and for
dependence of electron transport and diffusion in inner mem-
branes of varied protein density revealed a number of inter-
previably that there are no a priori reasons which prevent

time it takes to bring the reactive groups together by diffusion,
resulting in one collision causing one reaction. A diffusion
step is inclusive of the reorientation of approaching or op-
posed reactant molecules. Diffusion-based collisions of react-
tant molecules can be rate-limiting for a reaction, yet not be
at the theoretically limiting, diffusion-controlled rate in that
there may be more than one collision per reaction. Addition-
ally, others (43) have discussed why even a diffusion-con-
trolled reaction need not be inherently fast since, in principle,
such factors as orientation constraints can reduce and attrac-
tive forces can increase the association rate constant by
several orders of magnitude. These considerations are impor-
tant in cases of interacting biological macromolecules, where
steric hindrance, nonspecific binding, and internal modes of
motion come into play and where apparent association rates
can reach the diffusion limit (43). In this regard, we have
previously established definitions for 1) diffusion control, 2) diffusion as a rate-limiting step, and 3) reaction control for
the case of mitochondrial electron transport (3); and we have
pointed out that there are no a priori reasons which prevent
diffusion from being rate-limiting in electron transport.

Three lines of evidence presented in this study as well as
other data support diffusion as a rate-limiting step in the
ubiquinone region for maximum rates of electron transport.
1) The $E_a$ for the diffusion step alone was the most significant
contribution to the $E_a$ of the overall succinate-linked electron
transport processes for each protein density of the inner
membrane studied, i.e. native to 80% protein-diluted.
2) The $E_a$s for the succinate-linked electron transport activities
and for the diffusion step alone in the ubiquinone region both
decreased. This result indicates that as the resistance (effec-
tive viscosity – protein density) to diffusion in the inner
membrane is decreased, the resistance to electron transport
likewise decreases. Collectively and separately, these data are
compatible with diffusion-mediated collisions as a rate-limit-
ing step for maximum rates of electron transport.

DISCUSSION

The diffusion and kinetic measurements presented in this
study permit an assessment of the relationship between the
rate of mitochondrial electron transport and the rate of redox
component diffusion and collision in the ubiquinone region
of the electron transport sequence. Analyses of our results are
compatible with diffusion being a rate-limiting step in the
ubiquinone region for maximum (uncoupled) rates of electron
transport.

Diffusion as Rate-limiting Step in Mitochondrial Electron
Transport—A diffusion-controlled reaction is classically de-

defined as one in which the reaction rate is determined by the
time it takes to bring the reactive groups together by diffusion,
resulting in one collision causing one reaction. A diffusion
step is inclusive of the reorientation of approaching or op-
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and for the diffusion step alone in the ubiquinone region both
decreased in a similar, parallel fashion with decreasing inner
membrane protein density. 3) No significant temperature dependence for calculated collision efficiencies was noted as
indicated by Arrhenius plots of the ratio of predicted, theo-
retical collisions to productive collisions of Q with its redox
partners.

The first line of evidence that diffusion is rate-limiting in

![Fig. 4. Temperature dependence of electron transport and collisions/electron turnover in 30% protein diluted inner membranes.](image-url)
electron transport is based on the tenets of rate process theory (20, 21). Our findings that the $E_s$ for the diffusion step, i.e., the diffusion and collision of the redox partners, in the Complex II-Q-Complex III sequence constituted the most significant portion of the $E_s$ for each electron transport activity examined are consistent with diffusion being rate-limiting. The $E_s$ for the diffusion step as well as the overall kinetic activity were both normally high at ~12 kcal/mol for the native inner membrane. This is consistent with the effect of obstructed diffusion (13, 44), which causes significant resistance to long-range motion equivalent to a high effective solvent viscosity (13) and proportional to $1/D$. These findings are not in contradiction of, nor should they be confused with, the typically low $E_s$ expected for diffusion-controlled reactions that occur in dilute aqueous systems where the effective aqueous viscosities are normally very low compared to protein-dense biological membranes. The validity of our plots and our normally high values for the $E_s$ of diffusion in protein-dense membranes are strongly supported by studies of lateral diffusion in red blood cell membranes (45, 46). The $E_s$ for the actual process of electron transfer are low, i.e., 1–2 kcal/mol (47–50) compared to the overall kinetic process of electron transport in the intact inner membrane which includes the diffusion steps; therefore, it is unlikely that an electron transfer step would be rate-limiting. Collectively, these data support diffusion as a rate-limiting step in electron transport.

The second line of evidence that diffusion is rate-limiting in electron transport is based on the observed, parallel decreases in the $E_s$ for both the diffusion step in the ubiquinone region and the overall kinetic activity of electron transport as inner membrane protein density was decreased. These observations indicate that the factor that limits diffusion, i.e., the obstructive multicollisions due to the effects of protein density, limits the overall kinetic process of electron transport since the electron transport rate is coupled to diffusion (12, 13). That the membrane protein density has an effect on the temperature dependence of lateral diffusion was not unexpected despite the lack of systematic information on the temperature dependence of $D_s$ (51). Our $E_s$ for diffusion report the energy required for the physical process of motion (21) in inner membranes where obstructive multicollisions occur. Hence, viewed in terms of diffusion theory (44), the greater the extent of obstructive collisions, the greater the number of steps required to cover a given distance and the greater the $E_s$ for diffusion, as we found for the native compared to protein-diluted inner membranes. In terms of rate process theory (20, 21), the $E_s$ (the potential energy barrier) is related to the probability of finding a free volume for a diffusant to move into. The greater the protein density, the lower the probability of finding an adjacent free volume and therefore the higher the potential energy barrier ($E_v$). At higher temperatures, at a given protein density, more molecules have sufficient energy to overcome the potential barrier to diffusion, and there is a greater probability (21) of finding an adjacent free volume, thus, the greater the $D$.

The third line of evidence that diffusion is rate-limiting follows from the lack of any significant temperature dependence indicated by Arrhenius plots of the ratio of diffusion-based (theoretical) collisions to electron transport-based (productive) collisions for $Q$ with its redox partners. This analysis compares the ratio of collisions predicted by the Hardt equation (41) based on our measured $D_s$ to the actual diffusion-based collisions resulting in electron transfers. The above result is expected when diffusion is rate-limiting since any temperature effect on the diffusion step will be approximately the same for the overall, kinetic electron transport process, i.e., the increase in electron transport rate is virtually the same as that predicted by the increase in the rate of diffusion-based collisions as the temperature increases. This finding obtains irrespective of the Hardt reaction-diffusion equation used and the absolute rates of both turnovers and collisions since the Arrhenius treatment considers only the slope of the logarithms of the rates (i.e., log(predicted/actual collisions) versus $1/T$) as a function of temperature.

In light of these data, we conclude that diffusion is rate-limiting for maximal (uncoupled) rates of electron transport in the ubiquinone region of the electron transport sequence in the mitochondrial inner membrane. This finding is consistent with classically observed P pool kinetics and function as opposed to the case of absolute diffusion control (one collision = one reaction), which is thought to preclude such behavior (52). We have not, at this juncture, concluded that diffusion is rate-limiting for all respiratory states (e.g., coupled electron transport); however, we are currently examining this possibility.

Comparison to Related Studies on Ubiquinone-mediated Electron Transport—Other laboratories have reported results which are consistent with our conclusion that diffusion is rate-limiting in the ubiquinone region. For instance, electron transport rates were unaffected when the ubiquinone content of mitochondria in cultured, intact cells was decreased to ~60% of normal by inhibiting the biosynthesis of ubiquinone (53). Furthermore, studies on Rhodopseudomonas spheroides have shown a collisional interaction for quinone-mediated electron transport (54). In addition, the existence of quinone-mediated, diffusion-controlled electron transport has been established for some transbilayer electron transport reactions in model systems (55).

Our conclusions based upon FRAP measurements, set forth above and elsewhere (1–3, 12, 13, 29), regarding the rate and the role of ubiquinone diffusion in mitochondrial electron transport have been questioned by Lenaz and co-workers (56–58) based upon their fluorescence quenching measurements. The validity of our $D_s$ in the $10^{-9}$ cm$^2$/s range (2, 12, 13, 29) for ubiquinone in native inner membranes finds its basis in the type of measurement technique used, the diffusion distance relevant to the electron transport process, the norms for lateral diffusion in biological membranes, and membrane diffusion theory.

FRAP is currently the most widely used technique to directly determine $D_s$ and results using FRAP have been verified by comparison to other methods (3, 59). It directly measures the long-range (>1 μm) $D_s$ and intrinsically short-range $D_s$ of fluorocently labeled membrane components. Our findings for ubiquinone (2, 12, 13, 29) reveal an approximate 10-fold difference in $D_s$ in the native inner membrane (3.9 × 10$^{-10}$ cm$^2$/s) versus pure lipid bilayer (5.5 × 10$^{-9}$ cm$^2$/s) at 30°C. This difference is due to the multicollisonal obstructed nature of the long-range diffusion in the protein-rich native membrane and is predicted by the theory and experimental results of Eisinger et al. (44), who reported an approximate 10-fold decrease in $D_s$ for obstructed, long-range diffusion compared to both nonobstructed long-range and short-range (defined by Eisinger et al. to be <10 nm) diffusion. Thus, FRAP reports lateral diffusion inclusive of collisional interactions which are known to occur in the inner membrane and most significantly which are functionally essential to mitochondrial electron transport since electron transport is a multicollisonal process (13). Our earlier finding (2, 12, 29) that ubiquinone diffuses at the same rate as the phospholipids of the inner membrane bilayer in which it resides is consistent with diffusion theory (13, 44, 60, and references therein), with
the molecular composition of the inner membrane (7), and with the Ds of lipoidal molecules reported in the literature (3). The results of Ferguson-Miller *et al.* (30) comparing the diffusion of ubiquinone-10 labeled with NBD at the benzoquinone head group and shown to be internal in the membrane bilayer with NBD-phosphatidylethanolamine confirmed our earlier finding using Q,CO,NBDHEA (2, 29).

The fluorescence quenching technique used by Lenaz and co-workers (56-58) is an indirect method for estimating Ds that has recently been found to require further development ([cf. Ref. 61], and it measures only short-range diffusion (≤10 nm). Consequently, this technique is not, on average, sensitive to the functionally essential collisions of ubiquinone with its redox partners (or collisions with any integral proteins) in the inner membrane. Thus, Ds measured for Q analogues using this technique would be the same for inner membranes and asolectin phospholipid bilayers; and in the single experiment of Lenaz (57) and co-workers of a ubiquinone-3 in submitochondrial particles compared to asolectin, this was found to be the case. These two measurements and all their other measurements (56-58) for various Q analogues carried out exclusively in asolectin vesicles yielded Ds in the mid-10⁻⁶ cm²/s range, which are 2 orders of magnitude greater than our FRAP measurements of ubiquinone and DiI in dimyristoylphosphatidylcholine and asolectin and markedly higher than the Ds reported for all membrane lipoidal molecules in lipid bilayers by other investigators (3) using a number of techniques. Lenaz and co-workers (57, 58) contend that the high Ds they reported are due to a mid-bilayer location of Q; however, multicollisions between Q and the transmembraneous integral proteins of the inner membrane will occur regardless of the transbilayer location of Q, which effectively increases the resistance to motion for Q (13). In addition, it has been reported earlier (62, 63) that the benzoquinone head group of Q projects in an oscillatory manner toward both surfaces of the membrane; and therefore, Q must, on this basis alone, experience higher membrane viscosities than at the midplane of the bilayer.

A number of possible explanations for the unusually high Ds using fluorescence quenching reported by Lenaz and co-workers (56-58) can be given. 1) Anomalously high values can arise due to the contribution of static quenching, clearly evident as nonlinearities in the Stern-Volmer plots of Lenaz and co-workers (56) known to be a major factor with the quenching pair used (64). 2) Transient dynamic quenching gives rise to nonlinearities as well and hence to anomalously high Ds. In fact, this is reported to be the norm rather than the exception for membranes (61). 3) A recent numerical and experimental analysis (61) has shown that the Stern-Volmer quenching constant is a poor approximation due to its inappropriate use of the isotropic method and leads to overestimation of Ds up to orders of magnitude. 4) The technique requires corrections for the probe’s membrane partitioning behavior in order to determine the actual quenching constant and hence the D in the membrane. 5) Estimation of the quenching radius can affect the calculation of Ds by an order of magnitude (57). 6) Another uncertainty reported by Lenaz and co-workers (57) is the 5-fold discrepancy in D that results when the spin label 5-(N-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid is used in place of 16-(N-oxy-4,4-dimethyloxazolidin-2-yl)stearic acid. 7) Finally, measurement of short-range D which, on average, excludes the protein collisional component of lateral diffusion is irrelevant to the multicollisonal process of functional mitochondrial electron transport. Since it has been demonstrated (12, 13) that redox partners in the appropriate redox states collide more than once, on average, to successfully transfer electrons, it is essential to measure a D that includes the effects of all of the random collisional interactions that occur in the electron transport process and especially those reflecting collisions of the lipoidal ubiquinone with integral redox protein complexes.

Given the significant number of uncertainties in determining Ds by fluorescence quenching, we believe that future corrections will reach the values reported by us in pure lipid membranes using FRAP (2 × 10⁻⁶ cm²/s). For instance, based on the experimental work of Lenaz and co-workers (56-58) with asolectin vesicles, Blackwell *et al.* (61) have calculated a D of 2.6 × 10⁻⁶ cm²/s for ubiquinone-10 assuming a quenching radius of 1 nm. If we recalculate according to Blackwell *et al.* using 4 nm vis. Lenaz and co-workers (57), the resultant D for ubiquinone-10 in asolectin vesicles is 3.7 × 10⁻⁶ cm²/s. At this value for D, there would be essentially no discrepancy over the magnitude of the short-range D measurements using fluorescence quenching versus FRAP for Q in pure lipid bilayers. However, as we have pointed out, short-range Ds (estimated from lipid-lipid collisions) are basically insensitive to collisions of Q with any integral proteins, much less those functionally required collisions of Q with redox complexes.

In contrast to both our results and those of Lenaz’s group (56-58) has been the proposal by Hochman *et al.* (65, 66) of functional electron transport by multielectron-transferring “dynamic aggregates” of redox components. The original basis for this proposal was shown to be grounded on incorrect computations (3), which were recently revised (67), and are now compatible with the Random Collision Model. Although the Random Collision Model is not intended to absolutely rule out the possibility of occasional and statistically inconsequential short-lived contacts (aggregations), the existence of such aggregation currently lacks evidence and is unnecessarily speculative, considering the substantiated data on rates of diffusion.

In conclusion, the available evidence favors an electron transport, rate-limiting role in the inner membrane for the diffusion and collision of highly mobile redox components in the ubiquinone region, which is in concordance with the Random Collision Model of mitochondrial electron transport.

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