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Topical Review

Role of Mobility of Redox Components in the Inner Mitochondrial Membrane

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Introduction

Macromolecular interactions fall under two different categories (Berg & Von Hippel, 1985). In some cases the reactants are organized in a supramolecular framework, and their interaction is the result of either direct physical contact or a structural change bringing the reacting groups together within the supramolecular framework. In most cases, however, biological reactions occur by diffusion processes of one or both reactants in order to produce the interacting complex from the free components. The reduction of dimensionality from three to two dimensions, as it happens in membranes, is likely to improve the efficiency of diffusive reactions by enhancing the number of useful collisions between reactants. The recognition that biological membranes exist in a fluid state (Nicolson, 1976) and are endowed with relatively free mobility of lipids and of the molecules dissolved in the lipid bilayer (Van der Meer, 1984) represents a strong argument in favor of the importance of diffusion in membrane-associated macromolecular interactions.

In mitochondrial and other energy-conserving membranes, the mobility of the electron transfer components has drawn a special attention (Dixit & Vanderkooi, 1983) because of their crucial role in energy conservation.

Organization of Electron Transfer Chains

The original view on the organization of mitochondrial electron transfer equated the chemical sequence of individual components of increasing redox potential with a static physical sequence, the respiratory chain. The isolation in the laboratory of David Green of discrete lipoprotein redox complexes from the inner mitochondrial membrane and the finding that the respiratory chain could be reconstituted from the isolated complexes led Green (1966) to postulate that overall respiratory activity is the result of both intracomplex electron transfer in solid state between redox components having fixed steric relations and, in addition, of intercomplex electron transfer ensured by rapid diffusion of mobile components acting as cosubstrates, *vz.* ubiquinone (Q) and cytochrome *c* (cyt.*c*). The schematic organization of the respiratory chain is depicted in Fig. 1 and the properties of the respiratory components are summarized in Table 1.

Two extreme conditions can be envisaged for the structural organization of the respiratory chain (Rich, 1984; Lenaz & Fato, 1986) (Fig. 2): (i) The chain is organized in a liquid state; the large multiprotein complexes are randomly distributed in the plane of the membrane, where they move freely by lateral diffusion. Ubiquinone and cyt.*c* are also mobile electron carriers, whose diffusion rate is faster than that of the bulkier protein complexes; their diffusion-coupled collision frequencies may be either higher or lower than any given reaction step within the complexes, and consequently electron transfer would be either reaction limited or diffusion limited. (ii) Alternatively, the components of the chain are present as aggregates, ranging from small clusters of few complexes to the extreme of a complete solid-state assembly. The aggregates might be either permanent or transient, but their duration in time must be larger than any electron transfer turnover. The actual difference between the two models concerns the mechanism of electron transfer: in the random model, ubiquinone and cyt.*c*, by diffusing between complexes, ensure electron transfer at the time of each useful collision with them, whereas in the clustered model all redox reactions take place by direct electron transfer within the solid-state framework. It should be noted that

Key Words electron transfer · mitochondria · respiratory complexes · respiratory chain · organization · ubiquinone · diffusion · cytochrome *c* · diffusion · diffusion-limited

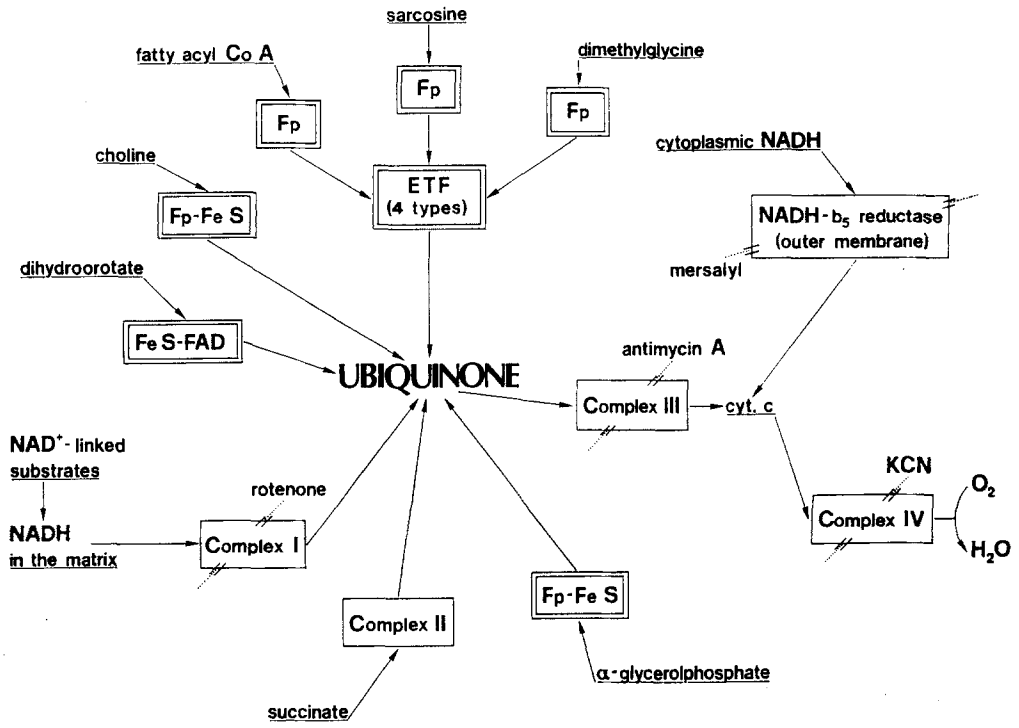

Fig. 1. Schematic picture of the mitochondrial respiratory chain of mammalian mitochondria

Table 1. Content and properties of some redox components of the inner mitochondrial membrane (bovine heart)[a]

Component	Concentration range		Molecular weight (kDa)	Number of polypeptides	Prosthetic groups
	(nmol/mg protein)	(μM in PL)[b,c]			
Complex I	0.06–0.13	0.12–0.26	700	26	FMN, 6–7 FeS
Complex II	0.19	0.38	200	4–5	FAD, 3 FeS
Complex III	0.25–0.53	0.50–1.06	250	10	2 b, c_1, FeS
Complex IV	0.6–1.0	1.2–2.0	160	12	a, a_3, 2 Cu
Cytochrome *c*	0.8–1.02	1.6–2.04	12	1	c
Ubiquinone-10	4	8	0.75	—	—

[a] Taken in part from Capaldi, 1982.
[b] Assuming phospholipids to be 0.5 mg per mg protein.
[c] Abbreviations: FeS, iron sulfur cluster; PL, phospholipids.

the latter model would be kinetically indistinguishable from the random model if Q and cyt.*c* associate to and dissociate from the complexes at rates higher than those of the redox reactions.

The random collision model has been systematically elaborated by Hackenbrock and his coworkers (Hackenbrock, 1981; Sowers & Hackenbrock, 1981; Schneider, Lemasters & Hackenbrock, 1982), who provided convincing evidence that under commonly employed experimental conditions all the respiratory chain components undergo independent lateral diffusion, so that electron transfer is a diffusion-coupled kinetic process. In the most recent version of their model, Hackenbrock, Chazotte and Gupte (1986) postulated also that electron transfer is rate limited by the diffusion of the faster components (ubiquinone and cyt.*c*).

The view of a solid-state arrangement of electron transfer components has presently fewer advocates; nevertheless, although the hypothesis of a whole supramolecular respiratory unit (Ozawa et al., 1986) is scarcely tenable, in view of kinetic and structural evidence to be discussed below, the possibility of transient aggregates (Hochman, Ferguson-Miller & Schindler, 1985) and preferential associations of the electron transfer components (*cf.*

Dixit & Vanderkooi, 1983; Gwak, Yu & Yu, 1986) is difficult to disprove.

In this review I will report on available evidence concerning the organization and mobility of electron transfer components in mitochondria and on the role of their diffusion in electron transfer, attempting, within the available knowledge, to discriminate possible differences existing between artificial or reconstituted systems and the native mitochondrial membranes *in situ*.

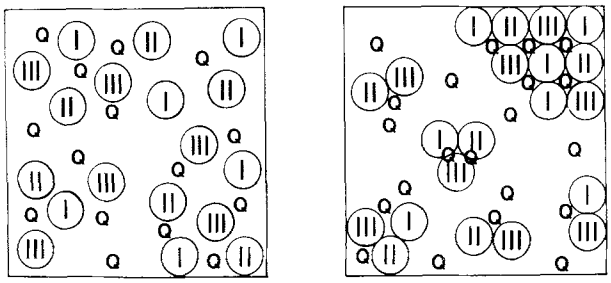

Fig. 2. Two models for the structural organization of the mitochondrial membrane in the ubiquinone region. *See* text for explanations. *I, II, III* are the corresponding complexes

Mobility of the Mitochondrial Membrane Components

Theoretical Aspects

The mobility of membrane-bound molecules appears essential for many biological functions, and the two-dimensional movement of proteins in biomembranes is a key feature in many membrane-associated functions (Axelrod, 1983; McCloskey & Poo, 1985).

A hydrodynamic model for membrane diffusion was given by Saffman and Delbrück (1975) for a cylindrical object, as a protein, embedded in a viscous continuum fluid sheet bounded by an aqueous fluid. Such a particle is restricted to move laterally in the x plane and to rotate around the z axis. Assuming viscosity of the membrane η to be much higher than viscosity of the outer medium η', the following equations were derived for lateral diffusion Dl and for rotational diffusion Dr, respectively:

$$\mathrm{Dl} = (kT/4\pi\eta h)(\ln \eta h/\eta' a - \gamma)$$
$$\mathrm{Dr} = kT/4\pi\, a^2 h$$

where k is Boltzmann's constant, T is absolute temperature, h is the membrane thickness, a is the radius of the cylinder, and γ is Euler's constant (0.5772).

The lateral diffusion of lipids and of hydrophobic molecules in the bilayer is not expected to depend on viscous drag from the outer medium, and the free-volume theory seems to apply best (Vaz, Goodsaid-Zalduondo & Jacobson, 1984); according to this theory (Montroll, 1969), the diffusion of a molecule includes the formation of local free volume by density fluctuations of the solvent cage, the subsequent jump of the diffusing molecule in the void, and the filling of the hole by another solvent molecule. It is expected that the lateral mobility of amphipathic molecules is determined by the free area in the outer polar region, whereas nonpolar molecules diffuse according to the freedom in the central region (Lenaz & Fato, 1986), which is more fluid (Seelig & Seelig, 1980), with higher probability of forming free volumes. Berg (1983) provided equations relating viscosity and molecular dimensions to diffusion of molecules of different shapes.

The method of choice for measuring lateral diffusion of proteins is fluorescence recovery after photobleaching (FRAP) (Peters, 1985); the method involves photochemical bleaching of a chromophore in a small region of the membrane with a strong impulse of laser excitation transmitted through a microscope; as diffusion occurs, the fluorescence intensity of the area increases and diffusion coefficients are calculated from the recovery curves. FRAP is only suited to measure long-range (>1 μm) diffusion: the possibility that biomembranes are laterally inhomogeneous (Curatola & Lenaz, 1987) can make the values of long-range diffusion quite different from those of short-range diffusion (Lenaz & Fato, 1986). Since chemical reactions and collision-dependent interactions are more directly related to local diffusion, this represents a real shortcoming of the method for the interpretation of such processes. The study of short-range diffusion involves determination of collisional encounters between two molecules, as EPR line broadening of spin labels, NMR, pyrene excimer formation, fluorescence collisional quenching, and others (*cf.* Lenaz & Fato, 1986); these techniques are widely used for lipids and small molecules but have not yet been extended to proteins.

Dl of lipids usually range between 10^{-7} and 10^{-9} cm^2/sec (Galla et al., 1979; Lenaz, 1988), appearing to fit the free area theory (Vaz & Hallman, 1983), and are slightly affected by the density of proteins in the membrane, only within the theoretical increase of viscosity induced by increased protein concentration (Peters & Cherry, 1982; Schindler, Osborn & Koppel, 1980*b*). Contrary to lipids, the diffusion coefficients of proteins range in a much broader field, between 10^{-8} cm^2/sec and complete immobilization (Cherry, 1979; Lenaz, 1988).

The Saffmann-Delbruck model has been tested by studying the mobility of bacteriorhodopsin inlayed in lipid bilayers (Peters & Cherry, 1982), which was confirmed to depend on viscosity of the outer phase; however, at very low lipid/protein ratios Dl decreased more than expected by the theoretical viscosity increase, suggesting other complications as the crowding effect due to hindrance to lateral movement by the concentrated protein dispersion (Saxton, 1982; Eisinger, Flores & Petersen, 1986).

In natural membranes the mobility of proteins does not follow the theoretical behavior; the observation that protein mobility depends on the type of membrane and of protein and is usually lower than theoretically expected suggests the existence of physiological restrictions that cannot be solely ascribed to the high protein density, including the cytoskeleton and the aqueous matrix in general (Schindler, Koppel & Sheetz, 1980*a*; Koppel, Sheetz & Schindler, 1981), regions of membrane junctions (Edidin, 1982), and protein aggregation (Barisas, 1984).

Diffusion of Mitochondrial Complexes

The ultrastructural study of Fleischer, Fleischer and Stoekenius (1967) on the fine structure of lipid-depleted mitochondria showed that the microscopic appearance of the inner membrane did not change after lipid removal, indicating that protein-protein contacts are sufficient to keep the membrane *in situ* as a result of its very high protein/lipid ratio. The high protein/lipid weight ratio, however, does not mean that there is a high protein/lipid area ratio, since most protein complexes completely span the lipid bilayer and extend beyond it on both sides, so that the actual membrane area occupied by phospholipids is rather high (50–60%) (Hackenbrock et al., 1986). Differential scanning calorimetry, detecting the thermotropic behavior of lipids removed from direct contact with proteins, showed that the majority of phospholipids in the inner mitochondrial membrane behaves as a free bilayer (Höchli & Hackenbrock, 1979). Freeze-fracture electron microscopy showed that the intramembrane particles are randomly distributed in the inner membrane (Sowers & Hackenbrock, 1981); although this can be taken as evidence for a random distribution of protein complexes in the lipid bilayer, it is not possible from microscopy alone to attain quantitative evaluations of the relative area of the membrane occupied by proteins and lipids. Additional evidence for a random distribution of electron transfer complexes stems from the fact that antibodies against Complex III and Complex IV aggregate these complexes separately (Hackenbrock & Hammon, 1975).

On the other hand, circumstantial evidence against a random distribution of respiratory complexes comes from the isolation of Complex I-Complex III (Hatefi, Haavik & Rieske, 1962) and Complex II-Complex III subcomplexes (Yu, Yu & King, 1974; Yu & Yu, 1980) and also of $b_6\gamma$-photosystem I units (Boardman, 1971), indicating that such units may be preferentially associated in the native membrane.

Obviously the problem is more rigorously attacked by direct investigation of the mobility of the mitochondrial components.

The lateral diffusion of protein complexes in mitochondrial membranes was measured by Sowers and Hackenbrock (1981) by a combination of post-field relaxation and freeze-fracture electron microscopy to quantitate the distribution of intramembrane particles, yielding Dl of 8.3×10^{-10} cm^2/sec for the particles in spherical mitoplasts of rat liver mitochondria; later, Gupte et al. (1984) reported values near 4×10^{-10} cm^2/sec using FRAP on labeled complexes I–IV in fused megamitochondria, whereas Hochman et al. (1985), also using FRAP, obtained Dl = 1.5×10^{-10} cm^2/sec for cytochrome oxidase in megamitoplasts from cuprizone-fed mice (*cf.* Table 2). The discrepancy between the electrophoretic relaxation method and FRAP may be accounted for by the fact that in the former a particle gradient is established, leading to possibly faster diffusion (Small et al., 1984). The independent diffusion of respiratory complexes and ATPase has been taken by Slater, Berden and Herveijer (1985) as evidence for a collision model for energy conservation through local proton gradients established by direct communication of respiratory complexes and ATPases during collisional encounters.

The diffusion coefficients of integral proteins protruding into aqueous compartments are strongly affected by the viscosity of the aqueous matrices (Hughes et al., 1982); mitoplasts lose substantial portions of matrix proteins (Caplan & Greenwalt, 1966), which in vivo form a quasi-solid lattice, possibly interacting with the integral membrane proteins (Srere, 1982); thus, the true long-range diffusion coefficients in intact mitochondria in vivo could be significantly lower than those measured in vitro. On the other hand, the long-range diffusion measured by FRAP could be slower than short-range diffusion, as the result of the high density of proteins. Accordingly, the diffusion coefficient of Complex III was strongly enhanced by the phospholipid enrichment of the membrane (Sowers & Hackenbrock, 1985; Hackenbrock et al., 1986); sig-

Table 2. Diffusion coefficients of some mitochondrial membrane components

Component	System	Technique	Dl × 10^9 (cm^2/sec)	Reference
Complex I	Fused mit.[a]	FRAP	0.4	Hackenbrock et al. (1986)
Complex II	Fused mit.	FRAP	0.4	Hackenbrock et al. (1986)
Complex III	Fused mit.	FRAP	0.44	Hackenbrock et al. (1986)
Complex IV	Fused mit.	FRAP	0.37	Hackenbrock et al. (1986)
	Megamit.	FRAP	0.15	Hochman et al. (1985)
Cytochrome *c*	Fused mit (μ = 0.3)	FRAP	0.06	Hackenbrock et al. (1986)
	Fused mit. (μ = 56)	FRAP	1.9	Hackenbrock et al. (1986)
	F.M.mit. (μ = 50)	FRAP	0.33	Vanderkooi et al. (1985)
	Megamit. (μ = 8)	FRAP	0.35	Hochman et al. (1985)
	Megamit. (μ = 25)	FRAP	0.74	Hochman et al. (1985)
Q-2 derivative	Fused mit.	FRAP	3	Hackenbrock et al. (1986)
Q-10 derivative	Fused mit.	FRAP	3	Ferguson-Miller et al. (1988)
	Lipid vesicles	FRAP	10	Ferguson-Miller et al. (1988)
Q-3	Lipid vesicles	F Q	6000	Fato et al. (1986)
Q-10	Lipid vesicles	F Q	2000	Fato et al. (1986)
	Lipid vesicles	F Q	400	Lenaz et al. (1988)[b]
Q-3	Mit. membranes	F Q	3000	Fato et al. (1986)
PQ	Lecithin vesicles	F Q	200	Blackwell et al. (1987)
PQH_2	Lecithin vesicles	F Q	200	Blackwell et al. (1987)

[a] Abbreviations: D_l, lateral diffusion coefficient; FRAP, fluorescence recovery after photobleaching; FQ, fluorescence quenching; mit., mitochondria; F.M.mit, flight muscle mitochondria; μ, ionic strength, mM; PQ, plastoquinone; PQH_2, plastoquinol.
[b] Recalculated from Fato et al. (1986) using the method for bidimensional collisional quenching reported by Blackwell et al. (1987).

nificantly, the long-range diffusion coefficient of phospholipids, though increased, was affected to much lesser extent.

The evidence for a random distribution of mitochondrial complexes, freely mobile in the inner membrane, is further supported by studies of rotational mobility (Dixit & Vanderkooi, 1983), showing that the rotational correlation time of cytochrome oxidase is the same whether complex III and cytochrome *c* are present or not in the same reconstituted system (Kawato et al., 1981).

In the native mitochondrial membrane, however, part of the cytochrome oxidase appears strongly immobilized (Kawato et al., 1981, 1982); Hackenbrock et al. (1986) ascribe the low mobile fraction of cytochrome oxidase to the use of a high sucrose solution; according to the Saffman Delbrück theory, however, only lateral diffusion, and not rotational diffusion, is affected by viscosity of the outer medium. Thus sucrose *per se* should not be responsible for the immobile fraction found in rotational diffusion experiments. The question still appears unresolved in terms of physiological activity, since the concentrated sucrose solution may well mimick the high viscosity of the fluids bathing the membrane, specially on the matrix side (Srere, 1982). In my opinion, the crucial question—whether mitochondrial complexes are immobile or mobile under physiological conditions in vivo—still awaits direct demonstration.

Diffusion of Ubiquinone

Gupte et al. (1984) measured the diffusion coefficient of a fluorescent derivative of a decyl-ubiquinone analog by FRAP, reporting Dl of 3×10^{-9} cm^2/sec; the same coefficient was found by Ferguson-Miller et al. (1988) using a fluorescent derivative of Q-10. On the other hand, from collisional quenching of membrane-bound fluorophores by ubiquinone homologs, Fato et al. (1986) calculated diffusion coefficients of $>10^{-6}$ cm^2/sec in both liposomes and mitochondrial membranes. By a modified Stern-Volmer relation for two dimensions, Blackwell et al. (1987) calculated coefficients of $>10^{-7}$ cm^2/sec for plastoquinone in lipid vesicles. Using this relation, Lenaz et al. (1987) recalculated coefficients of 4×10^{-7} cm^2/sec on their previous experiments (Fato et al., 1986) (Table 2).

The differences between Dl measured by the two methods are very large and probably to be ascribed to their different ranges of measurement. While in mitochondrial membranes a discrepancy of 2–3 orders of magnitude is largely to be ascribed to the high protein density, in lipid vesicles there is still a difference of 10-fold between FRAP and fluorescence quenching in measuring Q diffusion. This discrepancy might result from either the type of quinone, membrane inhomogeneities, incorrect use of dimensionality of the system or inadequacies of the equations used (Lenaz et al., 1988). Ubiquinone

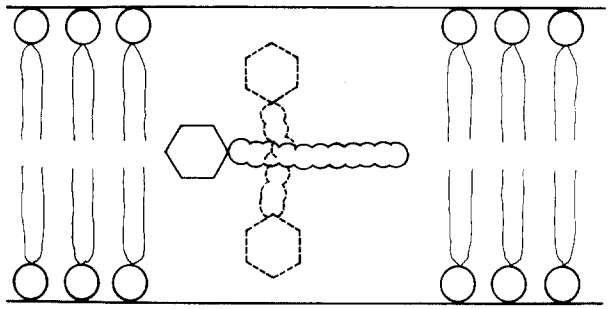

Fig. 3. A model for the localization of ubiquinone-10 in the lipid bilayer

diffusion measured by fluorescence quenching is not affected by the viscosity of the outer medium (Fato et al., 1986, 1987), in accordance with its location in the hydrophobic core of the membrane (Degli Esposti et al., 1981; Kingsley & Feigenson, 1981; Stidham, McIntosh & Siedow, 1984; Lenaz & Degli Esposti, 1985; Michaelis & Moore, 1985; Ulrich et al., 1985; Ondarroa & Quinn, 1986; Cornell et al., 1987), and then not subjected to drag from the outer medium (Vaz et al., 1984).

Our data support a model where most of the ubiquinone molecule is located in the membrane midplane, with the headgroup oscillating transversally across the membrane (*cf.* also Battino, Fahmy & Lenaz, 1986). The transversal movement of the ubiquinone molecule is most likely to be limited to oscillations of its relatively polar headgroup, allowing interactions with water-soluble redox agents (*cf.* Futami, Hurt & Hauska, 1979; Lenaz et al., 1977) (Fig. 3). In view of these considerations, the loss of respiratory activity induced by high sucrose (Hackenbrock et al., 1986) cannot be ascribed to any decrease of ubiquinone diffusion and must depend on other effects of sucrose on the respiratory enzymes.

Mobility of Cytochrome *c*

The lateral diffusion of cyt.*c* in mitochondrial membranes at low ionic strength is around 10^{-10} cm^2/sec (Hochman et al., 1982; Gupte et al., 1984; Vanderkooi, Maniara & Erecinska, 1985), but dramatically increases at higher ionic strength (Table 2); cyt.*c* is a peripheral protein, electrostatically bound to the outer surface of the inner membrane, from which is dissociated at high ionic strength (MacLennan, Lenaz & Szarkowska, 1966). Depending on ionic strength, cyt.*c* diffuses laterally in two dimensions on the membrane surface or in three dimensions within the intermembrane space (Hackenbrock et al., 1986) as also confirmed by resonance energy transfer to measure the distance of heme *c* from a fluorescent probe incorporated in the bilayer of the inner membrane, and showing an increase of distance from 4.3 nm at zero ionic strength to >10 nm at high ionic strength. No immobile fraction of cyt.*c* was found at high ionic strength by Gupte et al. (1984), whereas Vanderkooi et al. (1985) found that a fraction of 3 nmol of cyt.*c* derivative per mg protein remained immobile and tightly bound to the membrane. Dixit et al. (1982) found that a phosphorescent cyt.*c* derivative rotates at the same slow rate as cytochrome oxidase bound to mitochondrial membranes, suggesting complexation between the two molecules; the smaller radius of cyt.*c*, in fact, would make its rotation much faster if the molecule were completely free.

No doubt, cyt.*c* can undergo different mobilities depending upon experimental conditions. The question then becomes which conditions more closely resemble the physiological state of the molecule in the cell *in situ*. The problem will be further discussed on functional ground in a later section.

Mitochondrial Electron Transfer Is Coupled to Ubiquinone Diffusion

The idea that ubiquinone functions as a mobile electron carrier was supported by the kinetic analysis of the rate of electron input to ubiquinone (NADH-Q reductase, succinate-Q reductase, etc.) (Vred) and of electron output from reduced ubiquinone (ubiquinol oxidase) (Vox) in uncoupled submitochondrial particles. Under a wide range of input and output rates, Kröger and Klingenberg (1973*a*) established that ubiquinone distributes electrons randomly among the dehydrogenases and the bc_1 complexes, behaving as a freely diffusable intermediate. In fact, the observed electron transfer rate (Vobs) follows a hyperbolic relation:

$$\text{Vobs} = \text{Vox} \cdot \text{Vred}/\text{Vox} + \text{Vred}.$$

This expression, known as the homogeneous pool equation, was confirmed in a variety of experimental situations and is in line with the sigmoidal inhibition of electron transfer by antimycin (Kröger & Klingenberg, 1973*b*); in fact, onset of inhibition of succinate cyt.*c* reductase takes place only when Vox is inhibited to a level lower than Vred.

If the Q concentration is not saturating for the activity of the reducing and oxidizing enzymes, the equation is modified (Ragan & Cottingham, 1985), taking into account Q concentration, the individual V_{max} of the complexes and their dissociation constants for ubiquinone. Vobs is hyperbolically re-

lated to Qt and maximal turnovers of electron transfer are attained only at Qt saturating both Vred and Vox. Indeed the physiological concentration of Q-10 is in the range of the K_m of the Q-reactive enzymes; the K_m of the bc_1 complex for ubiquinol-10 is 3.3 mM in the phospholipids (Lenaz & Fato, 1986), as calculated from values of Zhu et al. (1982), and the K_m of Complex I for ubiquinone-10 is ca. 10 mM (Norling et al., 1984), while Qt in the inner membrane is in the range of 8 mM (corresponding to 4 nmol/mg protein). This means that ubiquinone is not saturating for either Vred or Vox. A double reciprocal plot of Vobs *vs.* Qt extrapolates to Vobs (max) of 28.5 nmol · sec^{-1} · mg^{-1} and a "*Km*" for Q-10 in NADH cyt.*c* reductase in bovine heart mitochondria of 7.5 mM in the phospholipids (Fig. 4); at physiological Q-10 concentration, the observed NADH cyt.*c* reductase activity would be about 50% of V_{max}.

The relation between electron transfer rate and Q concentration is seen in reconstituted systems and in phospholipid-enriched mitochondria for NADH oxidation (Schneider et al., 1982; Parenti Castelli et al., 1987, 1988; Fato et al., 1987); although NADH oxidative activities higher than the physiological rate can be attained, theoretical Vobs (max) has not been experimentally reached. The reason could be in the limited miscibility of ubiquinone with phospholipid bilayers; two-phase systems are formed just above the physiological Q concentration (Kingsley & Feigenson, 1981; Stidham et al., 1984; Ulrich et al., 1985; Ondarroa & Quinn, 1986); clustered ubiquinone would be kinetically inactive.

A similar behavior was found in photosynthetic electron transfer between reaction center and bc_1 complex in bacterial chromatophores (Casadio et al., 1984; Snozzi & Crofts, 1984). By investigating the second-order reduction of cytochrome b561 in chromatophores by the quinol produced by photoreduction in the reaction center at different redox poise of the quinone pool, a substrate-like dependence of the rate on ubiquinol concentration was found with an apparent K_m for ubiquinol-10 of 7 mM in the phospholipids (Venturoli et al., 1987).

Pool behavior contrasts the results of Maltese and Aprille (1985) showing that succinate oxidation is not decreased by lowering the Q content of cultured neuroblastoma cells by mevalonin administration; however, the K_m for ubiquinone-10 of succinate-Q reductase is much lower than that of NADH-Q reductase (Norling et al., 1974). Circumstantial evidence against a random distribution of respiratory complexes comes from the isolation of supercomplexes showing preferential association properties, as discussed previously. More recently,

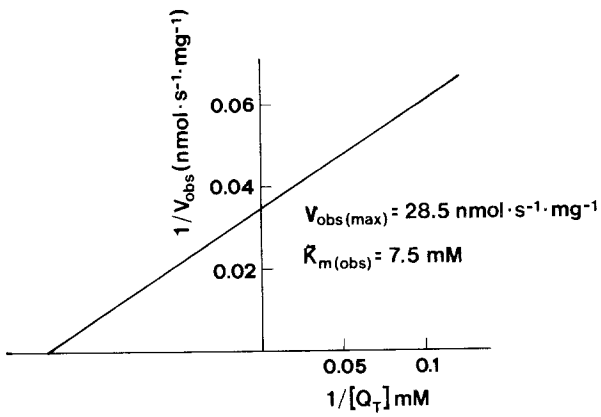

Fig. 4. Computed double reciprocal plot of NADH-cyt.*c* reductase (V_{obs}) in beef heart mitochondria, *vs.* the concentration of the Q pool (Q_t), according to Eq. (18) of Lenaz and Fato (1986) and using the following values: maximal velocities of Vred and Vox, respectively 45 and 78 nmol sec^{-1} mg^{-1}, K_m for Q reductase and QH2 oxidase, respectively 10 and 3.3 mM in the phospholipids

a differential scanning calorimetry study has indicated a preferential association of succinate-Q reductase (but not of NADH-Q reductase) to bc_1 complexes in vitro (Gwak et al., 1986). It is nonetheless evident that also succinate oxidation obeys to Q-pool behavior, as it is dependent on concentration of the Q-pool (Schneider et al., 1982; Lenaz et al., 1986).

It might be argued that Q-pool behavior was shown only in reconstituted systems or in mitochondria where the relations of the inner membrane complexes were altered by swelling (Schneider et al., 1982), freeze-thawing cycles (Parenti Castelli et al., 1987) or sonication (Kröger & Klingenberg, 1973*a*), whereas in intact mitochondria inner-outer membrane contacts and the quasi-solid organization of the matrix (Srere, 1982) may keep the integral proteins in a clustered immobilized arrangement. Nevertheless, it appears from the study of Stoner (1984) that intact coupled mitochondria in state 3 exhibit Q-pool behavior; strong evidence for a mobile intermediate between succinate and cyt.*c* was obtained by double inhibitor titrations. Figure 5 shows that inhibition of the bc_1 complex with myxothiazol under phosphorylating conditions makes succinoxidase less sensitive to the Complex II inhibitor 3'-hexylcarboxin, in accordance with the existence of a freely diffusable intermediate between the two steps (Baum, 1977).

It is our conviction that, even if Complexes II and III are closely associated in vivo in the inner membrane, electron transfer occurs only by collision and association to any molecule of Complex III

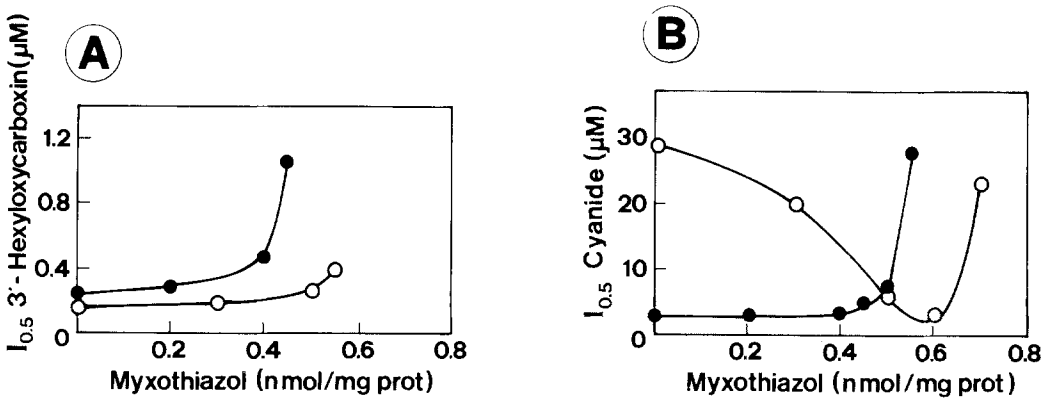

Fig. 5. Coupling relationship of succinate dehydrogenase to bc_1 complex (*A*) and of bc_1 complex to cytochrome oxidase (*B*) obtained by double inhibitor titrations. In *A* the half-inhibition ($I_{0.5}$) of succinate oxidase by the succinate dehydrogenase inhibitor 3'-hexylcarboxin was titrated against concentration of the bc_1 inhibitor, myxothiazol. In *B* the half-inhibition of succinate oxidase by the cytochrome oxidase inhibitor, cyanide, was titrated against myxothiazol concentration. ●—●, state 3 mitochondria; ○—○, uncoupled by S-13. Redrawn from Stoner (1984) with permission of the publisher. An increase of $I_{0.5}$ indicates presence of a diffusable intermediate, while a decrease indicates a fixed relation

of quinones reduced by any molecule of Complex II; the physiological significance of the preferred association between the two types of complexes is therefore unclear.

Q-pool behavior does not exclude the existence of an aliquot of quinone molecules, which are not freely diffusable but tightly bound to the complexes. Evidence for bound quinones (Suzuki & Ozawa, 1984) and for Q-binding proteins within the complexes (Yu & Yu, 1980, 1986; King, 1985) has been accumulating; no direct binding studies to the complexes were, however, reported for natural quinones. A fluorescence quenching method was applied in our laboratory to evaluate ubiquinone binding constants to the bc_1 complex inlayed in liposomes (Samworth, Degli Esposti & Lenaz, 1988); from modified Stern-Volmer plots of intrinsic tryptophan fluorescence quenching by Q-2, dissociation constants in the millimolar range were calculated, expressed as Q concentration in the membrane phospholipids. These high K_d's are not indicative of strong binding, and argue against the existence of Q-binding proteins; the bound quinone could be in rapid equilibrium with the pool, and therefore kinetically indistinguishable from it. Clearly, binding studies with the more hydrophobic natural Q-10 must be performed. Yu and Yu (1981) claimed to have demonstrated the existence of permanently bound quinone, with no exchange between free and bound forms. Such quinone should be distinguishable kinetically; no kinetic evidence, however, exists for participation of bound quinone in intercomplex electron transfer. The notion of a special bound quinone (Qz) in the bc_1 complex of bacterial chromatophores (Crofts et al., 1983) was abandoned in favor of a Qz site where the quinone pool rapidly exchanges (Snozzi & Crofts, 1984).

Does bound quinone, however, participate in intracomplex electron transfer?

It was shown by presteady-state kinetics that only a fraction of total ubiquinone is reduced by exogenous quinols as fast as cytochrome *b* (Van Hoeck et al., 1987). On the other hand, Q depletion to less than 0.1 Q per c_1 has no effect on the rate of cytochrome *b* oxidation by cyt.*c* in the prereduced complex in an antimycin-sensitive fashion (Degli Esposti et al., 1986). This result is incompatible with the Q-cycle (Mitchell, 1976) and with any cyclic scheme so far proposed in the complex (Rich, 1986), where the only oxidant allowed for cytochrome *b* was postulated to be either ubiquinone or ubisemiquinone. The cyclic pathways of electron transfer in the bc_1 complex are largely supported by experiments with use of specific inhibitors, particularly the so-called "double-kill," i.e., by combined center *i* and center *o* inhibitors, as antimycin plus myxothiazol (Berry & Trumpower, 1985). The extreme possibility raised by these findings is that cyclic electron transfer is an artifact produced by the inhibitors (Lenaz et al., 1985*a*), well known to strongly alter the conformation of the complex (Rieske & Ho, 1985). We postulate that ubiquinone is required for the bc_1 complex merely as a reductant. The pool in the lipid phase represents the free substrate and the bound ubi(semi)quinone the enzyme-substrate (transition) complex; the bound substrate should not be considered as a permanent prosthetic group involved in the intracomplex mechanism of electron transfer from a different exogenous ubiquinol molecule.

The Puzzle of Cytochrome c Mobility

The role of cytochrome *c* mobility between bc_1 complex (cytochrome *c* reductase) and Complex IV (cytochrome *c* oxidase) is even more intriguing than that of ubiquinone. The kinetics of electron transfer from cyt.*c* through the oxidase to molecular oxygen exhibit two or three phases (Thompson, Suarez & Ferguson-Miller, 1982); the polarographically determined rate of O_2 reduction is much faster than the net rate of spectroscopic appearance of oxidized cyt.*c* (Ferguson-Miller, Brautigan & Margoliash, 1978), suggesting that the initial rapid phase is due to multiple turnovers of bound cyt.*c* prior to dissociation from the oxidase. Consistently, a phosphorescent cyt.*c* derivative rotates at the same slow rate as cytochrome oxidase bound to mitochondrial membranes (Dixit et al., 1982), suggesting complexation, and cyt.*c* covalently bound to either the reductase or the oxidase is still capable of mediating electron transfer (Erecinska, Davis & Wilson, 1980; Waring et al., 1980); a mechanism of rapid oscillation of cyt.*c* between reductase and oxidase could explain fast respiratory rates. Hackenbrock et al. (1986) severely criticized the above interpretation on the basis that under physiological conditions of 150 mM ionic strength, cytochrome *c* is readily dissociated from the membrane and appears free to undergo three-dimensional diffusion (Gupte et al., 1984) in the intermembrane space; accordingly, duroquinol oxidase activity is enhanced by increasing ionic strength in parallel with the diffusion coefficient of cyt.*c*. Although cyt.*c* tends to collide with the membrane surface randomly, it may be guided electrostatically as it approaches the electric field of the membrane integral complexes (Koppenol & Margoliash, 1982).

In reconstituted systems of reductase plus oxidase in presence of excess cyt.*c*, pool behavior is followed (Froud & Ragan, 1984); likewise, the rotation rates of cytochrome oxidase reconstituted in lipid vesicles are not influenced by simultaneous incorporation of the reductase (Kawato et al., 1981), suggesting that the two enzymes and cyt.*c* are diffusing as independent entities. It is, however, to be considered that the concentration of cyt.*c* in the native membrane is roughly stoichiometric with the enzymes, yet sufficient to elicit full activity of the respiratory chain (Nicholls, 1976). Reconstitution of electron transfer complexes involves substitution of artificial lipids, disruption of matrix and intermembrane connections and possibly breakdown of original associations; no wonder if the mode of action of cyt.*c* in the native membrane could be widely different. It was reported that when cyt.*c* is used in amounts stoichiometric with cytochrome oxidase in intact mitochondria, its diffusion is insensitive to ionic strength (Maniara, Vanderkooi & Erecinska, 1984). Stoner (1984) could not clearly establish the existence of a diffusable intermediate between cyt.*c* reductase and oxidase in intact mitochondria, in contrast with the clearcut result in the ubiquinone region (Fig. 5).

Is Electron Transfer a Diffusion-Limited Process?

If the rate of a chemical reaction is limited by the time it takes to bring the reactive groups together via diffusion, the reaction is diffusion controlled; on the contrary, if subsequent chemical steps are limiting, the rate is reaction controlled (Berg & Von Hippel, 1985). Diffusion-limited reactions are viscosity dependent and have weak temperature coefficients. The association rate constant k for two spherical molecules A and B is

$$k = 4\pi N' D_{AB} r_{AB}$$

where N' is Avogadro's number per millimole, D_{AB} is the sum of the diffusion coefficients and r_{AB} is the sum of the radii of the two molecules. If the association reaction includes a chemical step, the rate constant k will be expressed by

$$1/k = 1/4\pi Dr + 1/k_c$$

where k_c is the rate constant of the chemical step. Normally it is assumed that k_c is viscosity independent; then a plot of $1/k$ *vs.* η is a straight line with intercept at $\eta = 0$ giving the inverse of the chemically controlled rate constant; if this intercept is close to zero, $k_c \gg 4\pi Dr$ and the reaction is diffusion limited. However, changes in solvent environment can also alter other parameters of the system, including macromolecular conformation (Beece et al., 1980) and energy transfer from the solvent to the active site (Somogyi, Welch & Damjanovich, 1984).

Several enzymic reactions occurring in membranes proceed at considerable rates, suggesting diffusion control. The possible presence of diffusion-limited components in enzymic reactions occurring in membranes has attracted considerable interest; for a diffusion-limited reaction, it was proposed (Adam & Delbrück, 1968) that reduction of dimensionality to two dimensions, as in membrane-mediated reactions, enhances the rate constants by facilitating collisional encounters. In fact, the diffusional search for a small target is much more efficient in two dimensions, assuming comparable values of the diffusion coefficient, because the

time to hit the target is proportional to the ratio distance/diameter of the target in three dimensions, but to the logarithm of this ratio in two dimensions. McCloskey and Poo (1985) calculated the two dimension *vs.* three dimension efficiency of a reaction such as that of cyt.*c* with cytochrome oxidase; since protein diffusion coefficients in membranes are much slower than in aqueous solutions, it comes out that the rate in the membrane is actually slowed down!

There may be, however, other reasons favoring bidimensional reactions in membranes. First, most cellular compartments are crowded with proteins, making up a gel-like viscous network where diffusion could be severely limited; furthermore, although protein diffusion in membranes is slow, hydrophobic substrates in the lipid bilayer (as quinones) may move fast, providing efficient collisional encounters; finally, rotational motion of integral membrane proteins is restricted to an axis normal to the plane, aligning the reactive groups in a way that is impossible for soluble macromolecules; the reduction of dimensionality of rotational diffusion to one dimension will be more advantageous than the reduction of dimensionality of lateral diffusion to two dimensions (McCloskey & Poo, 1985).

Diffusional Control of Individual Redox Reactions

In a study of solubilized cytochrome oxidase using media of different viscosities, Hasinoff and Davey (1987) found that the interaction of reduced cyt.*c* with the enzyme is partially diffusion-controlled. At high ionic strength, the minimum association rate constant $k_{min} = k_{cat}/K_m$, receives contributions from both diffusion control and chemical activation control, being about equal at a viscosity of *ca.* 5 cP. At low ionic strength the faster-reacting high affinity site is ~80% diffusion controlled, while the low affinity site is largely chemically controlled. Since the viscosity in the cytoplasm (and possibly in the intermembrane space, where cyt.*c* is physiologically contained) may range from 2 to 70 cP (Wojcieszyn et al., 1981) it was concluded that under physiological conditions the contribution of diffusion control to the activity of cytochrome oxidase may be quite significant. The steric probability factor for the interaction of the protein active site was low, with a probability for cyt.*c* to undergo a productive collision with the active site of the oxidase of one every 200–10,000 collisions.

We have investigated the interaction of ubiquinols with membrane-bound bc_1 complex (Parenti Castelli et al., 1987; Lenaz et al., 1988). The diffusion-limited collisional frequency of ubiquinol with the complex calculated using the Smoluchowski relation or analogous two-dimensional equations (Kano et al., 1981) is $>10^8$–10^9 M^{-1} sec^{-1} using as Dl for ubiquinol the short range value of 10^{-7}–10^{-6} cm^2/sec.

Ubiquinol cyt.*c* reductase activity in mitochondrial membranes is necessarily investigated using short chain ubiquinol homologs (Degli Esposti & Lenaz, 1982), which dissolve in the membrane by partitioning from the water phase before reaching the active site. Performing saturation kinetics at different membrane fractional volumes in the medium, Fato et al. (1988) devised a novel method to calculate the true Michaelis constants expressed as substrate concentrations in the membrane; the true $K_{min} = k_{cat}/K_m$ were in the range of $(1.8 \times 10^4$ M^{-1} sec^{-1} for ubiquinol-1 and $(6.3 \times 10)^4$ M^{-1} sec^{-1} for ubiquinol-2. Direct evaluation of the second-order rate constant of ubiquinol oxidation by presteady-state experiments was in excellent agreement with the k_{min} from the steady-state experiments (Lenaz et al., 1988). The only rate constant available for the natural ubiquinol-10, measured in bacterial chromatophores after flash activation of the reaction center, is $(3 \times 10)^5$ M^{-1} sec^{-1} (Crofts, 1986). These values are orders of magnitude smaller than the diffusion-controlled collisional frequencies; thus, either the process is reaction controlled or it is diffusion controlled with very low collisional efficiency. It is believed that collisional efficiencies are high in membranes, owing to the reduced dimensionality of lateral and rotational diffusion (*cf.* above).

The dependence of membrane-bound ubiquinol cyt.*c* reductase on the viscosity of the aqueous medium, varied by different polyethylene glycol concentrations, was investigated using short-chain ubiquinols as substrates (R. Fato and G. Lenaz, *unpublished*); the viscosity dependence of k_{cat}/K_m gave a negligible diffusion-limited component for ubiquinol-1, whereas 85–100% of the reaction rate was limited by cyt.*c* diffusion already at 1 cP viscosity (Fig. 6). The K_m increase of the enzyme for cyt.*c* at increased viscosity, concomitant with a K_m decrease for ubiquinol is typical for an enzyme using two substrates limited by diffusion of one of them (cyt.*c*) (Engasser & Hisland, 1978). The diffusion-limited fraction with cyt.*c* is higher in ubiquinol cyt.*c* reductase than in cytochrome oxidase, probably because the former has a greater turnover number (>100 sec^{-1}) than the latter (13 sec^{-1} in the report of Hasinoff and Davey (1987)). Assuming a diffusion-limited collisional frequency of $(1 \times 10)^{10}$ M^{-1} sec^{-1} for cyt.*c* and the reductase, as for reduced cyt.*c* and the oxidase (Hasinoff & Davey, 1987), the steric probability factor for interaction

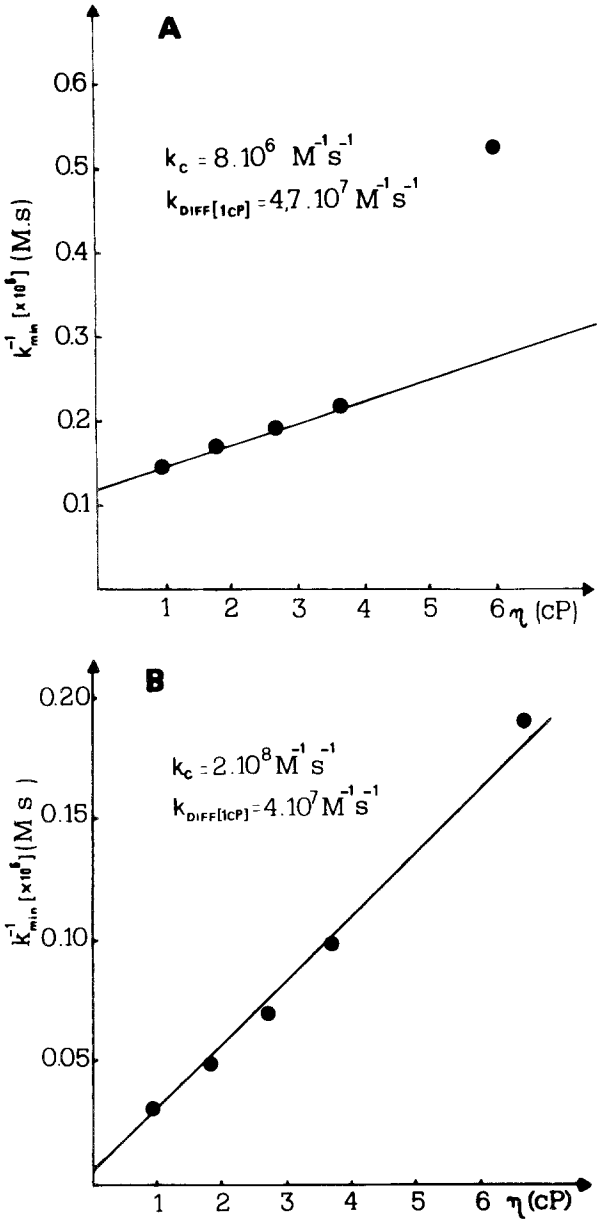

Fig. 6. Plots of k_{min} *vs.* viscosity in ubiquinol cyt.*c* reductase in submitochondrial particles. (*A*) Ubiquinol-1 titration at fixed cyt.*c* (*B*) Cyt.*c* titration at fixed ubiquinol-1. *See* text for explanations. Viscosity was varied by polyethylene glycol

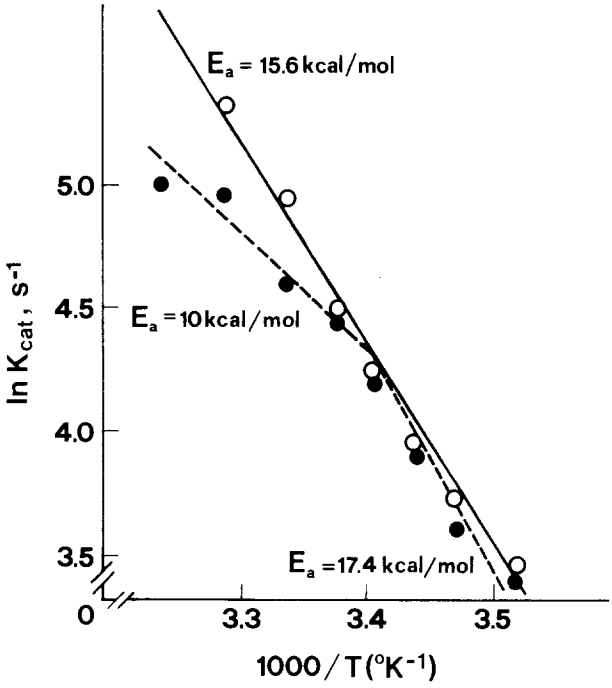

Fig. 7. Arrhenius plot of ubiquinol-2 cyt.*c* reductase activity in the isolated bc_1 complex inlaid in liposomes in presence and absence of cholesterol. ●—●, asolectin; ○—○, asolectin/cholesterol 2 : 1 (molar ratio)

was less than 1 every 100 collisions. The very strong viscosity dependence of cyt.*c* interaction with the bc_1 complex makes it unlikely that the reaction is concomitantly also limited by diffusion of ubiquinol, either in the water or in the membrane phase, even at the viscosities of 5–20 cP (Pedersen & Cox, 1986; Parenti Castelli et al., 1987) existing in the bilayer midplane.

The presence of diffusional limitations for water-soluble substrates in immobilized enzymes has been widely investigated; diffusional limitations in unstirred layers or in the matrix of immobilized supports lead to nonlinear saturation kinetics (Campbell & Hornby, 1975; Lluis, 1984), K_m increase for the more limiting substrate accompanied by K_m decrease for the other (Engasser & Hisland, 1978) and discontinuous Arrhenius plots with decreased activation energy at high temperature (Goldstein, 1976). All of these properties have been found in ubiquinol cyt.*c* reductase (*cf.* Degli Esposti & Lenaz, 1982; Parenti Castelli et al., 1987; Lenaz et al., 1988); in particular, the discontinuous Arrhenius plots are a characteristic feature of membrane-bound enzymes (Raison, 1972; Lenaz, 1979; Lenaz & Parenti Castelli, 1985). Other possible reasons for breaks in Arrhenius plots are, however, changes in rate-limiting step in the chemical reaction path, temperature-dependent conformational changes, phase changes of the phospholipids or viscosity becoming rate limiting for conformation flexibility (Lenaz & Parenti Castelli, 1985).

The activation energies (E_a) of ubiquinol cyt.*c* reductase *in situ* or in liposomes are 8–10 kcal/mol above and 18 kcal/mol below the break (at ca. 20°C) (Lenaz et al., 1986) (Fig. 7), whereas E_a of short-range ubiquinone diffusion investigated by fluorescence quenching was ca. 2–3 kcal/mol (Fato et al.,

Table 3. Average distances between mitochondrial redox complexes

Redox complexes	Total concentration of redox complexes (molecules/cm² membrane) × 10^{-10}		Minimum distance between complexes (nm)[b]	
	A. From Hackenbrock et al. (1986)	B. Calculated from Table 1[a]	Calculated from A	Calculated from B
Complex I	2.77	2.6–5.2	30	19–27
Complex III	8.12	10.8–22.8		
Complex II	5.35	8.2	27.2	18–22
Complex III	8.12	10.8–22.8		
Complex III	8.12	10.8–22.8	19.3	12–16
Complex IV	18.8	25.8–43.9		

[a] Assuming phospholipids with an average molecular weight of 750 kDa to occupy an area of 70 Å²/molecule (corresponding to 70 Å²/two molecules in a lipid bilayer). The values are minimal and maximal concentrations calculated from those in Table 1.
[b] Calculated by the formula $(c_1 + c_2)^{-1/2}$, where c_1 and c_2 are the concentrations of the two partner complexes in molecules/cm² membrane.

1986). Higher values (9–12 kcal/mol) were reported for long-range ubiquinone diffusion by FRAP (Hackenbrock et al., 1986). In the case of an individual enzyme there is no doubt that only short-range diffusion of its substrate is meaningful. It appears from the large difference existing between the E_a of short-range diffusion and that of ubiquinol cyt.*c* reductase activity that the collision-limited rate constant is much greater than the observed association rate constant. Wang, Berry and Crofts (1987) reached the same conclusion for ubiquinol cyt.c_2 reductase of Rps. sphaeroides.

Cholesterol incorporation in the membrane, enhancing bilayer viscosity and lowering Dl of ubiquinones (Fato et al., 1986) did not decrease ubiquinol cyt.*c* reductase activity, either k_{cat} or k_{cat}/K_m (Fato et al., 1987); the cholesterol level incorporated in bc_1 proteoliposomes (1 : 2 molar ratio with phospholipids) allows a uniform distribution of the sterol in the bilayer (Yeagle, 1985); it is therefore unlikely that the enzyme is confined into fluid patches of pure phospholipids.

Arrhenius plots of ubiquinol cyt.*c* reductase in cholesterol-enriched bc_1 proteoliposomes and submitochondrial particles revealed the disappearance of the characteristic break with increase of E_a above the breakpoint (Parenti Castelli et al., 1987) (Fig. 7), a behavior in line with that of other mitochondrial enzymes (Lenaz & Parenti Castelli, 1985). Note that a viscosity increase would enhance the diffusion-limited fraction in a partly diffusion-limited reaction, so that E_a should decrease, approaching that of diffusion.

The conclusion can be reached that ubiquinol cyt.*c* reductase may have a diffusion-limited component, but this component is present for aqueous diffusion of cyt.*c*, not for membrane diffusion of ubiquinone; the lower turnover numbers of the enzyme working in a concerted way in the respiratory chain make it even more unlikely that diffusion control by ubiquinone plays a significant role in electron transfer.

Diffusional Control of Integrated Electron Transfer

It may be reasoned that ubiquinone diffusion, though not rate limiting for the individual Q-reactive enzymes, becomes rate limiting in the integrated function of the Q pool, where the overall combined activity of two enzymes is constrained by the new parameter of intercomplex separation directing the reduced Q molecules toward Complex III and the oxidized ones back to Complex I or II; the diffusion path is run in a time proportional to the square of the intercomplex average distance (Berg & Purcell, 1977). Whereas in the individual enzymic activities short-range diffusion (<10 nm) is involved, in integrated electron transfer the diffusion process takes place on a average over a path of several nanometers. From the concentrations of electron transfer complexes in the mitochondrial membrane, average distances can be calculated through which the randomly distributed redox components must diffuse to effect a consecutive reduction and oxidation (Table 3).

A rough calculation indicates that in an area of 900 nm², scanned by a ubiquinone molecule leaving Complex I to reach Complex III, assuming a distance of 30 nm, there are about 12 protein molecules and 800 lipid molecules, if lipids occupy 60% of the total area. According to Eisinger et al. (1986) the long-range diffusion coefficients are slowed in proportion to the area covered by obstacles and to the reciprocal of their size. For a relative protein area

of 40%, with obstacles equated to hexagons having sides of length three times the lipid-lipid separation (equivalent to proteins having a radius of 2.4 nm), the diffusion coefficient of a molecule dissolved in the lipid phase would be lowered to 1/3 of the unobstructed value. Indeed, Hackenbrock et al. (1986) reported that lipid diffusion is enhanced about fourfold by a sevenfold phospholipid enrichment of mitochondrial membranes. On the contrary, Schindler et al. (1980), also using FRAP in *Escherichia coli* reconstituted membranes, found that on a range of protein concentration of 0–60% by weight, Dl for phospholipid remained essentially constant, whereas Dl for lipopolysaccharide decreased over 10-fold. Although the concentration and hence the cross-sectional area of integral proteins must be essentially similar in mitochondrial membranes and in *E. coli* reconstituted membranes, it appears from the two studies that phospholipid mobility is more severely affected by protein concentration in the former.

A possible explanation may lie in a stronger interference of the indocarbocyanine derivative used by Hackenbrock et al. (1986) with the peripheral portions of the proteins in comparison with the nitrobenzoxadiazole derivative used by Schindler et al. (1980*b*), in line with a lower Dl of the former (Derzko & Jacobson, 1980; *cf.* Table I in Hackenbrock et al., 1986) and with the Saffman-Delbrück dependence on viscosity of the outer medium. The same hypothesis would explain why diffusion of lipopolysaccharide, having a wide extramembranous moiety, is dramatically inhibited by increased protein concentration.

The uncertainties on the significance of protein crowding on obstruction of the diffusion path for small molecules like ubiquinone do not allow to predict from either short-range or long-range diffusion coefficients whether electron transfer in the inner mitochondrial membrane is diffusion controlled. Hackenbrock et al. (1986) approached the problem kinetically by comparing the temperature dependence of the overall steps (diffusion plus chemical reaction) in the Complex II-ubiquinone-Complex III span in the uncoupled inner membrane. The E_a for the overall diffusion steps for II-Q-III was calculated to be 12.2 kcal/mol, to be compared with E_a of 12.9 kcal/mol for succinate cyt.*c* reductase activity. The finding was interpreted as compatible with diffusion control of this electron transfer span; furthermore, when the protein/lipid ratio was decreased by phospholipid enrichment, the E_a's of both lateral diffusion and electron transfer decreased in proportion to the degree of enrichment. Similarly, they concluded that the rate-limiting step in duroquinol oxidase activity is the diffusion step of cyt.*c* to cytochrome oxidase. The increase of the rate of electron transfer catalyzed by cyt.*c* by increased ionic strength was taken to mean that the diffusional rate-limiting step of cyt.*c* is relieved by shifting from two-dimensional to three-dimensional diffusion.

The interpretation of studies concerned with activation energies is subject to considerable uncertainty, considering that most mitochondrial enzymes also not using ubiquinone, have a similar range of E_a (Lenaz, 1979). The E_a's of integrated electron transfer activities using the Q pool are usually within a close range to the E_a of ubiquinol-cyt.*c* reductase, with breaks in Arrhenius plots occurring at comparable temperatures (Lenaz, 1979). Evidence was presented in the previous section that ubiquinol cyt.*c* reductase is not controlled by the quinol substrate diffusion; likewise, the other individual enzymes using Q as substrate, having turnovers lower than that of the bc_1 complex, are not diffusion controlled. As it is unlikely that the diffusion step between Complex I or II and III has the same E_a of the individual enzymatic, chemically controlled activities, one would predict that, if the E_a of integrated electron transfer reflects a diffusion-limited step of the Q-pool, removal of this step by operation of the individual enzymes should lead to E_a's reflecting the chemical rate-limiting steps of those enzymes. Examination of the values from the literature (Table 4) does not support this idea; the E_a of ubiquinol cyt.*c* reductase is very close to those of succinate cyt.*c* reductase and of succinate oxidase. Moreover, Heron, Gore and Ragan (1979) in a reconstitution study of Complex I and Complex III found that operation of the Q pool slightly increased E_a of NADH cyt.*c* reductase above that of the same activity upon stoichiometric association of the complexes. Since the E_a's of NADH-Q reductase and NADH-cyt.*c* reductase are both higher than those of either succinate-cyt.*c* reductase and ubiquinol-cyt.*c* reductase, it appears unlikely that the same step (diffusion of either Q or cyt.*c*) is rate limiting for both NADH and succinate oxidation.

Although a diffusion-limited component may appear in the activity of individual enzymes working at vey high turnover numbers (*cf.* previous section for cyt.*c*), when electron transfer is integrated through a common substrate pool, the overall turnover is strongly decreased, reflecting the turnover of the slower enzyme, according to the pool equation (Kröger & Klingenberg, 1973*a*) and compensating the possible effect that the obligated distance between two complexes using a common intermediate (e.g. ubiquinone) imposes on the overall activity. Thus, the integrated reaction is not diffusion controlled.

The time t for a particle to diffuse to a small

Table 4. Activation energies of some mitochondrial enzymic activities

Enzyme	System	E_a (kcal/mol)[a]	Reference
Ubiquinol-1 cyt.*c* reductase	Soluble bc_1	17	This laboratory
Ubiquinol-2 cyt.*c* reductase	Soluble bc_1	18	This laboratory
Ubiquinol-3 cyt.*c* reductase	Soluble bc_1	16	This laboratory
Ubiquinol-2 cyt.*c* reductase	SMP[b]	8–18	This laboratory
	bc_1 liposomes	8–18	This laboratory
	Same, plus FCCP	11–18	This laboratory
	Same, plus cholesterol	16	This laboratory
NADH ubiquinone-2 reductase	Complex I liposomes	15–32	Poore & Ragan (1982)
Succinate cyt.*c* reductase	Mitochondria	13	Hackenbrock et al. (1986)
Succinate oxidase	Mitochondria	9–17	This laboratory
NADH cyt.*c* reductase	Proteoliposomes		Heron et al. (1979)
	Stoichiometric	15	
	Q-pool	17	
ATPase	SMP	12–22	This laboratory
Cytochrome oxidase	Mitochondria	7–15	This laboratory
Ubiquinone diffusion	Liposomes	3	This laboratory
	Mitochondria	2	This laboratory
	Mitochondria	12	Hackenbrock et al. (1986)
	PL-enriched mito.	9	Hackenbrock et al. (1986)

[a] Values approximated as integers in all cases. When two values are present, they represent E_a above and below the break, respectively.

[b] Abbreviations: E_a, activation energy; FCCP, *p*-trifluoromethoxy-carbonylcyanide phenylhydrazone; PL, phospholipids; SMP, bovine heart submitochondrial particles.

target of diameter d over a distance l in two dimensions is given by (Berg & Purcell, 1977)

$$t = (l^2/2D)(\ln l/d - 0.75).$$

For a distance of 30 nm, taking Dl of ubiquinone = $(4 \times 10)^{-7}$ cm^2/sec, and assuming a diameter of the active site of complex III of 1 nm, the time for a Q molecule reduced by Complex I to reach Complex III would be 30 μsec; for a turnover of 100 sec^{-1} (i.e., 10 msec/turnover), close to the physiological rate of NADH-cyt.*c* reductase, this time corresponds to over 300 collisions *with the active site* per turnover. The theoretical calculations show that the diffusion limit could be reached only at high turnovers of the chain or largely increased distances.

Assuming a random distribution of the complexes in the lipid bilayer, as controlled by freeze-fracture electron microscopy, increase of the phospholipid content with respect to protein is equivalent to increasing the average distance between complexes. Using this approach for Complexes I and III in reconstituted liposomes, Parenti Castelli et al. (1988) found that NADH-cyt.*c* reductase activity is not decreased by increasing intercomplex distance up to 108 nm, at an experimental maximal turnover of 50 sec^{-1}. Using the Berg and Purcell relation, this turnover corresponds to a lower limit for Dl of $(1.1 \times 10)^{-8}$ cm^2/sec in a diffusion-limited reaction having a collisional efficiency of 100%. Also the experiments in phospholipid-enriched mitochondria (Schneider et al., 1982; Parenti Castelli et al., 1987, 1988) confirm that phospholipid enrichment has no effect on electron transfer provided that Q concentration is maintained constant by enriching the membranes with liposomes containing ubiquinone as well.

Conclusions

Membrane fluidity is a prerequisite to allow diffusion of proteins and other molecules in membranes; however, diffusion-coupled processes do not appear, in general, to be significantly diffusion controlled (Lenaz, 1988). The possible presence of a diffusion-limited step in cyt.*c* interaction with its partners, Complex III and cytochrome oxidase, may not be extended to the overall electron transfer chain; moreover, the interaction of cyt.*c* with its partners under physiological conditions may not be fully governed by diffusion. These considerations would actually explain why electron transfer chains, which need to be associated to fast redox reactions for efficient ATP synthesis, have evolved with lipophilic quinones as redox mediators between complexes of the respiratory chain. If electron transfer were associated with collisions of re-

dox proteins directly, their slow diffusion would be rate limiting. Lipophilic quinones, by virtue of their fast lateral diffusion in the lipid bilayer, meet the requirement of fast electron transfer limited only by the chemical processes within the complexes. In this respect, the changes in lipid fluidity induced by diet, cold acclimatation, etc., would not modify the rate of diffusion-coupled membrane processes, because Q diffusion is too fast to be rate limiting. On the other hand, stoichiometric associations, which can render electron transfer more efficient in the linear cyt.*c* region, are forbidden in the highly branched ubiquinone region where reducing equivalents are funneled by several dehydrogenases.

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