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The Role of Cytochrome *c* Diffusion in Mitochondrial Electron Transport*

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Sharmila Shaila Gupte and Charles R. Hackenbrock

From the Laboratories for Cell Biology, Department of Cell Biology and Anatomy, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

We have compared the modes and rates of cytochrome *c* diffusion to the rates of cytochrome *c*-mediated electron transport in isolated inner membranes and in whole intact mitochondria. For inner membranes, an increasing ionic strength results in an increasing rate of cytochrome *c* diffusion, a decreasing concentration (affinity) of cytochrome *c* near the membrane surface as well as near its redox partners, and an increasing rate of electron transport. For intact mitochondria, an increasing ionic strength results in a parallel, increasing rate of cytochrome *c*-mediated electron transport. In both inner membranes and intact mitochondria the rate of cytochrome *c*-mediated electron transport is highest at physiological ionic strength (100–150 mM), where the diffusion rate of cytochrome *c* is highest and its diffusion mode is three-dimensional. In intact mitochondria, succinate and duroquinol-driven reduction of endogenous cytochrome *c* is >95% at all ionic strengths, indicating that cytochrome *c* functions as a common pool irrespective of its diffusion mode. Using a new treatment to obtain bimolecular diffusion-controlled collision frequencies in a *heterogenous* diffusion system, where cytochrome *c* diffuses laterally, pseudo-laterally, or three-dimensionally while its redox partners diffuse laterally, we determined a high degree of collision efficiency (turnover/collisions) for cytochrome *c* with its redox partners for *all* diffusion modes of cytochrome *c*. At physiological ionic strength, the rapid diffusion of cytochrome *c* in three dimensions and its low concentration (affinity) near the surface of the inner membrane mediate the highest rate of electron transport through maximum collision efficiencies. These data reveal that the diffusion rate and concentration of cytochrome *c* near the surface of the inner membrane are rate-limiting for maximal (uncoupled) electron transport activity, approaching *diffusion control*.

Mitochondrial electron transport mediated by cytochrome *c* has been studied extensively (*e.g.* Refs. 1–6) and traditionally, cytochrome *c* and its redox partners have been viewed as a structurally stable macromolecular aggregate, comprising the heme protein portion of the electron transport "chain" (7, 8). However, using fluorescence recovery after photobleaching (9) and immunofluorescence imaging (10), we have determined that the electron transferring components of the mitochondrial inner membrane are independent lateral diffusants and, for a *homogenous* (two-dimensional) diffusion system, determined that ordered chains (7, 8), respiratory assemblies (11), or transient aggregates (12–14) of cytochrome *c* and its redox partners are not necessary for cytochrome *c*-mediated electron transport. We have concluded that the overall process of mitochondrial electron transport includes diffusion and random collisions of the redox components which represents the basis for the random collision model of electron transport (9).

In our preceding paper (15) we reported on the rates and modes (lateral, pseudo-lateral, and three-dimensional) of cytochrome *c* diffusion and calculated the collision frequencies of cytochrome *c* with its redox partners for each diffusion mode. In this paper, we compare the modes and rates of cytochrome *c* diffusion to the rates of electron transfer from cytochrome bc_1 to cytochrome oxidase in isolated inner membranes and in whole intact mitochondria at various ionic strengths. Using the kinetic data from this paper and new treatment to obtain collision frequencies in *heterogenous* diffusion systems (15), we calculate here the collision efficiencies (turnover/collisions) of cytochrome *c* with its redox partners. The collision efficiencies reveal that the diffusion rate and concentration (affinity) of cytochrome *c* near the surface of the inner membrane are rate-limiting for maximum (uncoupled) electron transport activity, approaching *diffusion control*. At physiological ionic strength, cytochrome *c* diffuses rapidly in a three-dimensional mode and has the lowest affinity for the inner membrane while mediating the highest rate of electron transport through maximum collision efficiencies with its redox partners.

EXPERIMENTAL PROCEDURES

Membrane Preparations—Rat (male, Sprague-Dawley) liver mitochondria were isolated and inner membranes (mitoplasts) were prepared using digitonin (16). The purified inner membranes were resuspended in H_{300} medium (300 mosM buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes,[1] pH 7.4).

Enzyme Assays—Duroquinol oxidase activity was measured polarographically with a Clark oxygen electrode (17) according to Schneider *et al.* (18). For an individual set of experiments, electron transport activity at each ionic strength was measured in triplicate and all samples in an individual set were statistically randomized. Three different sets using different inner membrane preparations were used to determine the duroquinol oxidase activity at each ionic strength. Nonenzymatic rates of electron transport were measured in the presence of 0.2 μg/ml antimycin A and 2 mM KCN added to the chamber prior to the addition of inner membranes. Eadie-Hofstee plots of duroquinol oxidase activity were analyzed using a two-component linear regression computer program. Succinate reductase and duroquinol reductase activities were measured using a SLM-

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; duroquinol, tetramethyl-*p*-benzoquinol.

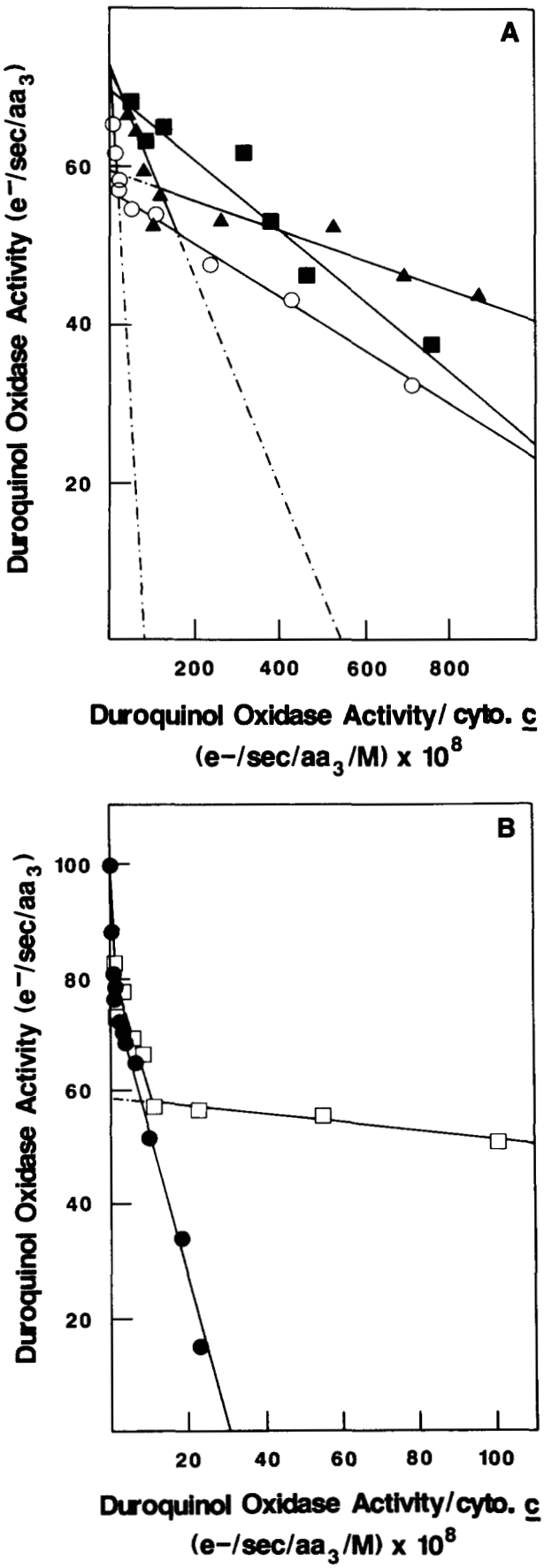

FIG. 1. **Eadie-Hofstee plots of duroquinol oxidase activity of inner membranes in KCl-H_{300} medium (300 mosM medium containing various concentrations of KCl and H_{300} medium, buffered with 2 mM Hepes, pH 7.4) in the presence of various concentrations of exogenous cytochrome c.** Other assay conditions were: 0.6 mM duroquinol, 1 μM carbonyl cyanide *m*-chlorophenylhydrazone, 5 μM rotenone, and 1.0 mM EDTA, pH 7.4. *A*, Ionic strengths: *a*, 0 (■), 25 (▲), and 50 mM (○); *B*, 100 (□) and 150 mM (●).

TABLE I

Ionic strength dependence of cytochrome c-mediated electron transport in inner membranes

Ionic strength	Duroquinol oxidase activity			
	High affinity		Low affinity	
	K_m (cytochrome *c*)	V_{max}	K_m (cytochrome *c*)	V_{max}
mM	*M*	e^-/*s*/*heme* aa_3	*M*	e^-/*s*/*heme* aa_3
0			3.4×10^{-10}	69.57
25	1.9×10^{-10}	59.42	1.3×10^{-9}	73.07
50	3.2×10^{-10}	56.77	9.1×10^{-9}	75.69
100	7.7×10^{-10}	58.48	2.2×10^{-8}	83.09
150	7.5×10^{-9}	75.21	2.4×10^{-7}	103.01

Aminco DW-2C spectrophotometer in dual wavelength mode.

Materials—Horse heart cytochrome *c* (type VI) and duroquinone were purchased from Sigma. All chemicals were reagent grade.

RESULTS

Characteristics of Cytochrome c-mediated Electron Transport in Isolated Inner Membranes—Maximum cytochrome *c*-mediated duroquinol oxidase activity and apparent K_m values, as a measure of the affinity of cytochrome *c* for its redox partners, were determined for inner membranes from 0 to 150 mM ionic strengths as utilized in our diffusion measurements of cytochrome *c* (15). Eadie-Hofstee plots of duroquinol oxidase activity (Fig. 1, *A* and *B*) revealed biphasic kinetics for cytochrome *c*-mediated electron transport at all ionic strengths except 0 mM (Table I). The high affinity K_m increased by an order of magnitude while the low affinity K_m increased by 4 orders of magnitude as the ionic strength was raised from 0 to 150 mM revealing a decrease in the affinity of cytochrome *c* for its redox partners with increasing ionic strength (Table I). These results for duroquinol oxidase activity in intact inner membranes are consistent with results obtained for purified beef heart ubiquinol-cytochrome *c* reductase (19) and cytochrome oxidase (20). The high affinity V_{max} remained relatively constant from 25 to 100 mM ionic strength, whereas the low affinity V_{max} increased with increasing ionic strength from 0 to 150 mM. Increases in the rate of electron transport mediated by cytochrome *c* occurred in both KCl/Hepes and KP_i buffers indicating that the ionic strength-dependent rate of electron transport is not due to an anion-specific effect. These data reveal that with increasing ionic strength the rate of electron transport mediated by cytochrome *c* increases and the affinity of cytochrome *c* for its redox partners decreases while, as we have shown (15), the diffusion rate of cytochrome *c* increases.

Cytochrome c-mediated Electron Transport in Isolated Inner Membranes Compared to Whole Intact Mitochondria—The duroquinol oxidase activities in both inner membranes and in intact mitochondria were measured over a broad range of ionic strengths (Fig. 2). Duroquinol oxidase activity of inner membranes, containing only endogenous cytochrome *c*, increased with increasing ionic strength up to approximately 50 mM but decreased with further increase in ionic strength. Similarly, in the presence of 20 μM exogenous cytochrome *c*, duroquinol oxidase activity in inner membranes increased with increasing ionic strength up to approximately 100 mM and decreased with further increase in ionic strength. At an

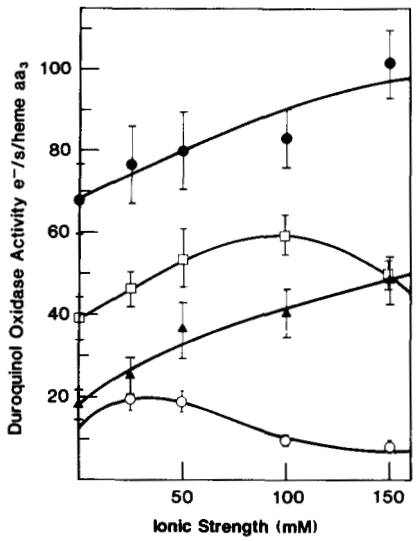

FIG. 2. **Duroquinol oxidase activity of inner membranes and intact whole mitochondria with increasing ionic strength.** Inner membranes with only endogenous cytochrome *c* (○); inner membranes in the presence of 20 μM exogenous cytochrome *c* (□); inner membranes in the presence of 100 μM exogenous cytochrome *c* (●); intact whole mitochondria with only endogenous cytochrome *c* (▲). Other assay conditions were: KCl-H_{300} solution at various ionic strengths, 0.6 mM duroquinol, 1 μM carbonyl cyanide *m*-chlorophenylhydrazone, 5 μM rotenone.

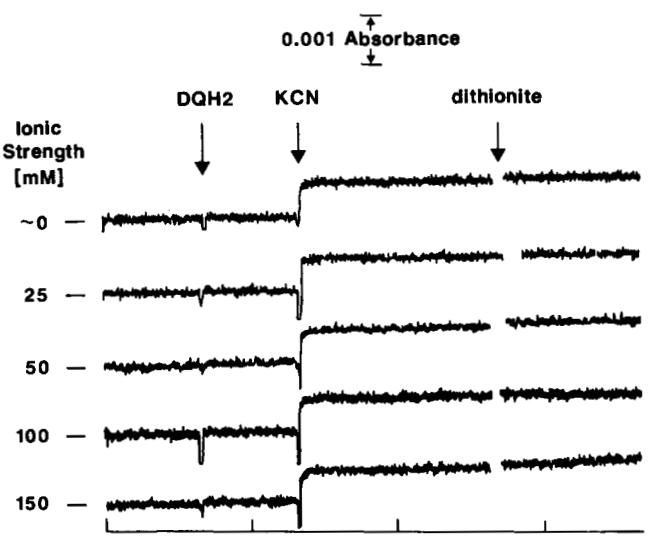

FIG. 3. **Reduction of endogenous cytochrome *c* in intact mitochondria at various ionic strengths.** The enzymatic reduction of cytochrome *c* was measured at 550–540 nm wavelength pair during duroquinol reductase activity. The assay conditions were: 60 μM duroquinol, 5 μM rotenone, 1 μM carbonyl cyanide *m*-chlorophenylhydrazone, 1 mM KCN. The total reduction of cytochrome *c* was measured by adding solid sodium dithionite.

exogenous cytochrome *c* concentration of 100 μM, which is equal to its physiological concentration in the intact mitochondria, a greater enhancement and progressive increase in duroquinol oxidase activity occurred in inner membranes as ionic strength was increased from 0 to 150 mM. Duroquinol oxidase activity of intact mitochondria paralleled this progressive increase in the rate of electron transport with increasing ionic strength and showed, as with isolated inner membranes, that cytochrome *c* mediates maximum electron transport in the intact mitochondria at physiological ionic strength (Fig. 2). Thus duroquinol oxidase activity is greatest in both isolated inner membranes and intact mitochondria at physiological ionic strength where we have shown (15) using inner membranes that cytochrome *c* diffuses in three dimensions.

Common Pool Function of Cytochrome c During Steady State Electron Transport in the Whole Intact Mitochondrion—Considering the increase in the duroquinol oxidase activity with increasing ionic strength, in intact mitochondria, the question arises, does *all* the cytochrome *c* of an intact mitochondrion participate in steady state electron transport at low ionic strength, when it is membrane-bound and diffusing laterally and at high ionic strength, when it is not membrane-bound and is diffusing in three dimensions? To determine whether cytochrome *c* functions in a common pool for all of its diffusion modes, we measured the percent reduction of endogenous cytochrome *c* in intact mitochondria between 0 and 150 mM ionic strength, using either succinate or duroquinol as substrate, followed by the addition of KCN (Fig. 3). It was determined that more than 95% of the cytochrome *c* was enzymatically reducible with either substrate compared to its total reduction by sodium dithionite for all ionic strengths studied. These data indicate that *all* of the endogenous cytochrome *c* in intact mitochondria participate in steady state electron transport irrespective of its diffusion mode.

DISCUSSION

Characteristics of Electron Transport in Isolated Inner Membranes Related to Cytochrome c Diffusion—Cytochrome *c*-mediated steady state duroquinol oxidase activity can be divided into four major distinct reaction (electron transfer) and diffusion steps: the reaction of oxidized cytochrome *c* with reduced cytochrome bc_1; the diffusion of reduced cytochrome *c* to oxidized cytochrome oxidase; the reaction of reduced cytochrome *c* with oxidized cytochrome oxidase; and the diffusion of oxidized cytochrome *c* back to reduce cytochrome bc_1. In these steps, the steady state reaction (electron transfer) rates of duroquinol reductase and cytochrome oxidase activity are 3–7 times faster than the overall duroquinol oxidase activity (19–21). The maximum reaction rate of the reduction of oxidized cytochrome *c* by reduced cytochrome bc_1 is 480–590 e^-/s (19) and the maximum rate of oxidation of reduced cytochrome *c* by oxidized cytochrome oxidase is even higher (20, 21). From these reaction rate data, it is clear that the slowest, or rate-limiting step, in the overall duroquinol oxidase activity is the transport of electrons between cytochrome bc_1 and cytochrome oxidase by cytochrome *c*, *i.e.* the diffusion step of cytochrome *c*.

To more directly ascertain the relationship of cytochrome *c* diffusion to the overall kinetic process of electron transport, we determined the apparent K_m values of cytochrome *c* and the maximum rates (V_{max}) of duroquinol oxidase activity in inner membranes at various ionic strengths. Our data showed a decrease in the affinity of cytochrome *c* for its redox partners and an increase in V_{max} for duroquinol oxidase activity when the ionic strength of the medium was increased from 0 to 150 mM. These findings are consistent with a decrease in the electrostatic interaction of cytochrome *c* for its redox partners and consequently a shorter binding interval of cytochrome *c* for the active sites of its redox partners as ionic strength is increased as predicted by theoretical calculations (22, 23). These data, combined with our diffusion and binding data (15), show that a decreased affinity and an increased rate of diffusion of cytochrome *c*, which occur as ionic strength is raised, results in an increased rate of electron transport.

Electron Transport and Cytochrome c Diffusion in Isolated Inner Membranes Compared to Whole Intact Mitochondria—

We determined that the duroquinol oxidase activity of inner membranes containing only endogenous cytochrome *c* increased with increasing ionic strength up to 50 mM. This can be correlated with the faster pseudo-lateral diffusion of cytochrome *c* at 50 mM compared to lower ionic strengths for inner membranes (15). At higher ionic strengths, endogenous cytochrome *c* leaves the surface of the inner membrane, consistent with our fluorescence recovery after photobleaching, resonance energy transfer by Förster mechanism, and direct binding data (15), to become so diluted that it is unavailable for electron transport. It is logical that in the intact mitochondria, cytochrome *c* leaves the surface of the inner membrane above 50 mM ionic strength. However, the maximum volume available for three-dimensionally diffusing cytochrome *c* in the intact mitochondria is limited by the ion-permeable outer membrane, thus, its duroquinol oxidase activity does not decrease with increasing ionic strength but rather increases. Our kinetic data (Table I, Fig. 2) on the duroquinol oxidase activity in inner membranes (Table I) indicates that this increase in the rate of cytochrome *c*-mediated electron transport with increasing ionic strength in intact mitochondria and in cytochrome *c* supplemented inner membranes as well, is due to three-dimensionally diffusing (low affinity) cytochrome *c* and not due to small quantities of membrane-bound (high affinity) cytochrome *c*. The increase in duroquinol oxidase activity with increasing ionic strength parallels an increase in the low affinity V_{max} for electron transport and a large increase in the low affinity K_m of cytochrome *c*. In contrast, the small increase in the high affinity K_m of cytochrome *c* and relatively constant high affinity V_{max} of electron transport indicate that electron transport cannot be maintained by small quantities of high affinity membrane-bound cytochrome *c*.

The parallel maximal duroquinol oxidase activity which occurs at physiological ionic strength in both inner membranes and in intact mitochondria, requires a naturally high concentration of cytochrome *c*. This finding suggests to us that the decrease in the electrostatic interaction of cytochrome *c* with the inner membrane at physiological ionic strength, which results in the three-dimensional diffusion of cytochrome *c*, is compensated for by the naturally high concentration of cytochrome *c* found in the intact mitochondria.

Pool Function of Freely Diffusible Cytochrome c—We reported cytochrome *c* to be freely diffusible at the inner membrane surface at all ionic strengths and for all modes of diffusion (15). In the present paper, we have shown that >95% of the cytochrome *c* in intact mitochondria is enzymatically reducible at *all* ionic strengths consistent with studies using single ionic strengths (24–26). These diffusion and enzymatic data indicate that all cytochrome *c* is freely diffusible in a common pool and participates in electron transport in intact mitochondria whether it is bound to the inner membrane surface and diffusing laterally at low ionic strength, or not bound to the inner membrane and diffusing in three dimensions at higher ionic strengths, including physiological.

A diffusible pool of cytochrome *c* has been proposed previously from kinetic studies of reconstituted ubiquinol oxidase (6). The mobility of cytochrome *c* has also been argued from a number of studies showing that the electron transfer interaction domains of cytochrome bc_1, cytochrome oxidase, and cytochrome b_5, are essentially the same, *i.e.* all bind to the same domain on the cytochrome *c* molecule (27–30). From these studies, it neither appears necessary nor indeed possible, for cytochrome *c* to interact simultaneously as a tertiary complex with its two redox partners during its electron trans-

TABLE II

Diffusion-based collision efficiency of cytochrome c-mediated electron transport

Redox partners	Effective quantities[a] (molecules/inner membrane)	Ionic strength	Diffusion rate[b]	Diffusion mode	Collision frequency	Collision frequency[d]	Experimental turnover number[e]	Collisions/ turnover[f]	% collision efficiency[g]
		mM	cm^2/s		*collisions/s/cm²*	*collisions/s/ redox partner*	*turnovers/s/ redox partner*		
Cytochrome bc_1 (reduced)	919	0	4.9×10^{-10}	Lateral	7.1×10^{14}	bc_1 54,700	985.0	55.6	1.8
Cytochrome *c* (oxidized)	15,209	50	2.3×10^{-9}	Pseudo-lateral	6.8×10^{14}	*c* 3,350	60.3	55.6	1.8
						bc_1 40,900	1,158.0	35.3	2.8
						c 2,510	70.9	35.3	2.8
		150	1.0×10^{-6}	Three dimensional	3.1×10^{14}	bc_1 23,900	1,470.0	16.3	6.1
						c 1,470	90.0	16.3	6.1
Cytochrome *c* (reduced)	1,880	0	4.3×10^{-10}	Lateral	8.7×10^{13}	*c* 3,310	481.1	6.9	14.6
Cytochrome oxidase (oxidized)	10,646	50	2.3×10^{-9}	Pseudo-lateral	8.7×10^{13}	aa_3 584	85.0	6.9	14.6
						c 3,260	566.9	5.7	17.4
						aa_3 575	100.0	5.7	17.4
		150	1.0×10^{-6}	Three-dimensional	7.9×10^{13}	*c* 3,000	719.4	4.2	24.0
						aa_3 528	126.9	4.2	24.0

[a] Quantities of reduced and oxidized redox partner molecules under conditions of maximum (uncoupled) electron transport calculated from the data of Klingenberg and Kroger (25) as in Gupte *et al.* (9).

[b] Combined diffusion coefficients of cytochrome *c* and its redox partners from Gupte and Hackenbrock (15).

[c] Diffusion-controlled collision frequency, calculated by the treatment of Astumian and Chock (31) as described in Gupte and Hackenbrock (15).

[d] Collision frequencies of one active redox molecule with its available redox partner calculated as in Gupte *et al.* (9).

[e] Maximum (uncoupled) duroquinol oxidase activity of inner membranes in the presence of 100 μM cytochrome *c* taken from Fig. 2. Duroquinol oxidase activity was 68 e^-/s/heme aa_3 at approximately 0 ionic strength; 80 e^-/s/heme aa_3 at 50 mM ionic strength; 101.5 e^-/s/heme aa_3 at 150 mM ionic strength.

[f] Calculated by column 7/column 8.

[g] Calculated by column 8/column 7.

fer. Our data are consistent with a common pool function in which diffusion-based collisions of cytochrome *c* with its membrane-restricted redox partners mediate electron transport.

Diffusion-based Collision Efficiency of Cytochrome c-mediated Electron Transport—We have used the treatment of Astumian and Chock (31) to determine the theoretical diffusion-based collision frequencies in a *heterogenous* diffusion system between potentially active cytochrome *c* and its redox partners, cytochrome bc_1 and cytochrome oxidase, in collisions/s/cm^2 of inner membrane (15). Using these collision frequencies (shown in Table II, column 6) we can derive the collision frequencies of one active redox molecule with its active redox partner (column 7), which can be compared to its electron transfer frequencies or turnover numbers (column 8) to obtain its collisions/turnover (column 9) and collision efficiencies (column 10) for all three diffusional modes of cytochrome *c*.

This new diffusion and electron transport data and treatment thereof (Table II) show that the collision frequencies (column 7) of cytochrome *c* with cytochrome bc_1 and cytochrome oxidase are closer to their respective experimentally determined electron transport frequencies (column 8) for *all* diffusion rates (column 4) and *all* diffusion modes of cytochrome *c* (column 5). With increasing ionic strength, the diffusion rate of cytochrome *c* increases (column 4), the collision frequencies of all redox partners decrease (column 7), their electron transport frequencies increase (column 8), and their collision efficiencies increase (column 10). Concomitantly, the affinity (from K_d, resonance energy transfer, and binding data) of cytochrome *c* for the inner membrane decreases with increasing ionic strength (15). At physiological ionic strength, where the diffusion rate of cytochrome *c* is maximal and three-dimensional, and its affinity for the inner membrane is lowest, its diffusion-based collision efficiency with either of its redox partners is highest, approaching values consistent with *diffusion control* (column 10).

These results underscore the contributions of both diffusion and concentration (affinity) of cytochrome *c* near the surface of the inner membrane in the overall electron transport process mediated by cytochrome *c*. As indicated in our companion paper (15), at lower ionic strengths, where the diffusion rate of cytochrome *c* is lower, its collision frequency with its redox partners is higher since its concentration near the inner membrane and its redox partners is also higher. At lower ionic strength, the occupancy time of cytochrome *c* in the vicinity of the active site of the redox partner before and after the transfer of an electron is greater. Thus for steric reasons, this decreases the probability for a subsequent, effective collision, resulting in a lower rate of electron transport, therefore, a lower collision efficiency. In contrast, at higher and physiological ionic strengths, where the diffusion rate of cytochrome *c* is higher and three-dimensional, its collision frequency with its redox partners is lower since its concentration near the inner membrane and its redox partners is also lower. At physiological ionic strength, the occupancy time of cytochrome *c* in the vicinity of the active site of the redox partner before and after the transfer of an electron is shorter. Thus, at physiological ionic strength there is a high probability for a subsequent, effective collision resulting in a higher rate of electron transport, therefore, a higher collision efficiency.

These findings are consistent with a conformational basis for association and dissociation of redox partners in the random collision model of mitochondrial electron transport. Redox proteins appear to exist in various conformations (32, 33). Thus, for any diffusional mode of cytochrome *c*, a productive, *i.e.* electron transferring, collision is thought to require a conformation-specific association between the active site of cytochrome *c* and its redox partners. It follows that a concerted conformational change occurs in the redox partners concomitant with electron transfer which, we believe, is the basis for an electron-induced, conformation-driven dissociation from the *active site* of such redox partners. This dissociation is required before a subsequent, diffusion-based, productive collision can occur.

In summary, our findings reveal that for any of the three diffusion modes of cytochrome *c*, the diffusion-based collisions of cytochrome *c* with its redox partners have a direct influence on the overall kinetic process of electron transport. Furthermore, our findings reveal that at physiological ionic strength, cytochrome *c* diffuses rapidly in three dimensions and has a low affinity for the inner membrane to mediate the highest rate of electron transport through maximum collision efficiencies with its redox partners. The collision efficiencies reveal that the diffusion rate and concentration of cytochrome *c* near the membrane surface are rate-limiting for maximal (uncoupled) electron transport activity, approaching *diffusion control.*

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