Multidimensional Diffusion Modes and Collision Frequencies of Cytochrome c with Its Redox Partners*

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We have determined the modes and rates of cytochrome c diffusion as well as the collision frequencies of cytochrome c with its redox partners at the surface of the isolated, mitochondrial inner membrane over a broad range (0–150 mM) of ionic strengths. Using fluorescence recovery after photobleaching, resonance energy transfer, and direct binding assay, we determined that the diffusion coefficient of cytochrome c is independent of its concentration and quantity bound to the inner membrane, that the distance of cytochrome c from the membrane surface increases with increasing ionic strength, and that there is no significant immobile fraction of cytochrome c on the membrane regardless of ionic strength. The rate of cytochrome c diffusion increases while its mode of diffusion changes progressively from lateral to three-dimensional with increasing ionic strength. At physiological ionic strength (100–150 mM), the diffusion of cytochrome c is three-dimensional with respect to the surface of the inner membrane with a coefficient of 1.0 × 10⁻⁶ cm²/s, and little, if any, cytochrome c is bound to the membrane regardless of its concentration. Furthermore, as ionic strength is raised from zero to 150 mM, the cytochrome c kₐ for the inner membrane increases, its mean occupancy time on the inner membrane to collide with a redox partner (r) decreases, and its diffusion-based collision frequencies with its redox partners decreases. These data reveal the significance of both diffusion and concentration (affinity) of cytochrome c near the surface of the inner membrane in the control of the collision frequency of cytochrome c with its redox partners.

N Meduch observations support the concept that electron transfer between specific, nonstoichiometric redox components of the mitochondrial inner membrane is coupled to, i.e., preceded by, random, diffusion-based collisions between these components (1–7). Applying the technique of fluorescence recovery after photobleaching (FRAP) to a homogenous (two-dimensional) diffusion system, we have previously shown that the redox protein complexes and ubiquinone, all integral to the inner membrane, diffuse laterally in the membrane plane at rates which are independent of ionic strength (7). In contrast to these redox components, cytochrome c is a basic redox protein that associates with the surface of the mitochondrial inner membrane (8, 9). At neutral pH and low ionic strengths, cytochrome c interacts electrostatically at the membrane surface with its redox partners, cytochrome bc₁ and cytochrome oxidase, as well as with membrane lipids (10). Thus, unlike other redox components, cytochrome c can be removed from the mitochondrial inner membrane simply by washing in 150 mM KCl, i.e. at physiological ionic strength (11). For these reasons, the physical and functional interactions of cytochrome c with the mitochondrial inner membrane are understandably complex.

In this report, we report results which reveal that the rate of cytochrome c diffusion and its diffusion mode vary with ionic strength but are independent of cytochrome c concentration irrespective of ionic strength. We also report on the relative binding and proximity of cytochrome c to the membrane as a function of ionic strength using a direct binding assay and resonance energy transfer by the Förster mechanism (RET) (12). The data show that cytochrome c functions as a three-dimensional diffusant at a physiological (100–150 mM) ionic strength. From these data, we present a new treatment to calculate the diffusion-based collision frequencies for a heterogenous diffusion system where cytochrome c diffuses laterally, pseudo-laterally, or three-dimensionally while its redox partners diffuse in two dimensions at various ionic strengths, including physiological. The data reveal the significance of both the diffusion and concentration (affinity) of cytochrome c near the inner membrane surface in controlling its collision frequency with its redox partners. In the accompanying article (46), we report on the rates of electron transfer mediated by cytochrome c for each of its three diffusion modes and present the significance of its three-dimensional diffusion mode in the electron transport process in isolated inner membranes as well as in intact, whole mitochondria at physiological ionic strength.

EXPERIMENTAL PROCEDURES

Mitochondrial Inner Membrane Preparation—Rat (male, Sprague-Dawley) liver mitochondria were isolated, and inner membranes (mitoplasts) were prepared using digitonin (13). The purified inner membranes were resuspended in H₂O medium (300 mM solution containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4).

FRAP and Binding Assays—As previously described (7), FRAP was carried out on KCl-washed, Ca²⁺-fused mitochondrial inner membranes in a glass chamber. In the first binding assay, the glass-attached membranes were labeled using 20 μl of various concentrations of FITC-cytochrome c in appropriate buffer. The quantity of FITC-cytochrome c bound to the glass-attached membranes was detected fluorometrically (7). In the second binding assay, 2 or 20 nmol of native or FITC-horse heart cytochrome c were added to KCl-washed membranes (5.0 mg/ml protein) in 0.3 mM Hepes (0.3 mM ionic strength), 10 mM KF, (23 mM ionic strength), and 25 mM KF.
(56 mM ionic strength) buffer in 1.0-m1 total volume, incubated at room temperature for 5 min, and then centrifuged in a Beckman Microfuge Model B for 4 min at 9000 x g. The pellets were solubilized with a final concentration of 0.8% sodium deoxycholate and 100 mM KP, pH 7.4. The cytochrome c content of the pellet material and supernatant was determined by obtaining the difference spectra of ferricyanide-oxidized versus dithionite-reduced cytochrome c.

Preparation of Assoiectin Vesicles—Assoiectin (1 g) was hydrated in 5 ml of H_2O, medium for 1 h on ice and then sonicated on ice for 30 min at 10-min intervals using a Branson sonicator at a power setting of 5 (output = 35 watts) to form small, unilamellar vesicles (14). These vesicles were centrifuged at 23,000 x g for 60 min to remove titanium pieces and large, multilamellar vesicles. The supernatant containing small, unilamellar vesicles was used within 48 h.

Incorporation of DPH into Mitochondrial Inner Membranes—The concentration of DPH in tetrahydrofuran was determined spectrophotometrically using an extinction coefficient of 60,000 M^-1 cm^-1 at 350 nm. DPH was added slowly in 1 ml aliquots to inner membranes while stirring. DPH in aqueous medium is nonfluorescent; therefore, it was unnecessary to remove unbound DPH. The conditions for the DPH incorporation into inner membranes were: DPH:phospholipid ratio, 1:100; concentration of the solvent tetrahydrofuran, <0.5%. Labeling was carried out at 0°C while stirring for 60 min. The succinate oxidase activity of DPH-labeled inner membranes was approximately 85% compared to unlabeled inner membranes. DPH-labeled inner membranes were stored overnight and used within 24 h after labeling.

RET Measurements—RET measurements were performed essentially as described by Gupta and Lane (15). The intensity of DPH emission incorporated into inner membranes was monitored digitally at 410 nm using a Perkin-Elmer fluorescence spectrophotometer 450-500 in the ratio mode at 23°C. The excitation wavelength for these measurements was 365 nm, and the slit width of both excitation and emission monochromators was 5 nm. For the determination of percent RET from membrane-bound DPH to cytochrome c as a function of ionic strength, KCI/H_2O medium (300 mM solution containing various concentrations of KCl and H_2O medium buffered with 2 mM Hepes, pH 7.4) was utilized. The percent RET was calculated as (1 - (fluorescence intensity of DPH at a given KCl concentration/fluorescence intensity of DPH at 150 mM KCl concentration)) X 100 in the presence of cytochrome c. For an individual set of experiments, the percent RET for each KCl concentration was measured in triplicate. For each cuvette, the digital output of fluorescence intensity was signal-averaged for 9 s. All samples in an individual set were statistically randomized. The distance (R) for 50% RET and the actual distance (R) = R as carried out at 0°C while stirring for 60 min. The spectral overlap integral (J) for this pair was calculated to be 7 x 10^-4, the refractive index was 1.4, and the orientation factor (K^2) was assumed to be 2/3 for the random orientation of donor and acceptor molecules. The quantum yield of DPH incorporated into the membrane was taken to be 0.8 (17).

The life-time of DPH was determined using a SLM 4800 phase modulation fluorescence spectrophotometer as described by Barrow and Lentz (18) using the following settings: excitation wavelength = 366 nm; emission monitored using a Schott KV 460 broad-band cutoff filter; slit width = 3 nm; and modulation frequency = 18 MHz. The decrease in the fluorescence life-time of membrane-bound DPH in the presence of cytochrome c was noted in H_2O medium, but not in 150 mM KCl. These observations were sufficient to verify that the decrease in the fluorescence intensity of membrane-incorporated DPH, only in H_2O medium in the presence of cytochrome c, was due to RET the Förster mechanism. Therefore, the two components of DPH life-time were not resolved. Since the life-time of DPH remained unchanged in the presence and absence of cytochrome c at 150 mM KCl, the fluorescence intensity of DPH at 150 mM KCl was used in the proximity studies as a reference, indicating no RET.

Materials—Horse heart cytochrome c (type VI) and FITC was purchased from Sigma; DPH was purchased from Molecular Probes (Eugene, OR); and tetrahydrofuran (gold label) was purchased from Aldrich. All chemicals were reagent-grade.

RESULTS

Membrane Binding of Cytochrome c—When FITC-labeled cytochrome c was eluted on a CM52 column (19), five major peaks were obtained. Addition of peak IV FITC-cytochrome, which eluted immediately prior to the native cytochrome c peak, to inner membranes resulted in same succinate oxidase activity as native cytochrome c. Thus, peak IV appeared to consist of cytochrome c with a FITC-labeled lysine which is not significantly involved in either the electron transport process or binding to its redox partners; hence, peak IV was selected for FRAP studies.

The quantity of FITC-cytochrome c bound to inner membranes was determined by two separate methods (Table I). The first method consisted of a fluorometric measurement of the percentage of the total FITC-cytochrome c bound to the membranes in the FRAP chamber. The second method consisted of incubation of either FITC-cytochrome c or native cytochrome c with the membranes and subsequent centrifugation, followed by spectroscopic measurement of cytochrome c bound to the pelleted membranes. The data from these two diverse types of assays show that the amount of FITC-cytochrome c and native cytochrome c bound to the membranes decreased to the same extent as the ionic strength of the medium was increased.

Lateral and Pseudo-lateral Diffusion of Cytochrome c—We have previously determined that the rate of lateral diffusion of cytochrome c on the inner membrane is ionic strength-dependent (7). In this study and contrary to the results of Vandenhoek et al. (20), we determined that the rate of lateral diffusion of cytochrome c at different ionic strengths <56 mM is independent of the quantity of cytochrome c bound to the membrane, including physiological, and its concentrations (Table I). In addition, in our study, the percent fluorescence recovery of FITC-cytochrome c after photobleaching indicated no significant immobile fraction of cytochrome c on the membrane surface at any ionic strength or concentration. Experiments designed to measure the FRAP of FITC-cytochrome c at ionic strengths >56 mM were not possible since, as with native cytochrome c, very few FITC-cytochrome c molecules remain bound to the membranes for sufficient times at these ionic strengths to be detected by FRAP.

Assessment of Cytochrome c-Membrane Proximity by RET—The proximity of cytochrome c to, or its average distance from, the membrane over a broad range of ionic strengths was determined by measuring the RET from the fluorescent probe DPH incorporated into the bilayer to the cytochrome c heme. DPH was utilized as a donor because of its ease of incorporation as well as high quantum yield in membranes (21). We determined the spectral overlap between DPH and the cytochrome c heme to be excellent and the extinction coefficient of the reduced and oxidized cytochrome c to be equal at 410 nm. Thus, the increase in intensity of DPH emission at 410 nm, using an excitation wavelength of 365 nm, was measured in the presence of cytochrome c at various ionic strengths to calculate the RET and hence, the average distance of cytochrome c from the membrane.

Initially, the RET from DPH, incorporated into the sonicated asolectin vesicle bilayer, to cytochrome c heme at various ionic strengths from 0 to 150 mM was measured to characterize the RET. We found the RET to decrease progressively with increasing ionic strength. The physiological concentration of free K^+ and Cl^- ions in the cytoplasm is 100-150 mM (22), which was accepted as the physiological ionic strength in our studies. The KCl concentration and ionic strength in the range of 0-150 mM KCl were treated as equivalent by assuming the activity coefficients of K^+ and Cl^- to be equal to 1. The change in RET was identical for KCl and NaCl at equivalent ionic strengths, which reveals that the change in the proximity of cytochrome c to the membrane is ionic strength-dependent and is not due to a specific ion.
average distance of cytochrome c strengths (Fig. 1). For this ratio, the RET decreased, and the c:phospholipid ratio, 1:83) was monitored at various ionic strengths in the presence of cytochrome c, which showed that the life-time of DPH incorporated into the vesicle bilayer is independent of ionic strength. In addition, it was determined that RET was independent of the DPH:cytochrome c ratio (data not shown).

Life-time of DPH in the Bilayer—The fluorescence life-time of DPH, measured for various conditions, was identical when measured at zero or 150 mM ionic strength in the absence of cytochrome c, which indicated that the life-time of DPH incorporated into the vesicle bilayer is independent of ionic strength (Table II). In addition, the life-time of DPH remained unchanged at 150 mM ionic strength when cytochrome c was present in the aqueous medium, indicating that no RET from DPH occurred. In contrast, the average life-time of the membrane-incorporated DPH decreased at zero ionic strength in the presence of cytochrome c, showing the presence of a short life-time component of DPH emission. The decrease in DPH life-time occurred at zero and not at 150 mM ionic strength in the presence of cytochrome c, showing the presence of a short-life-time component of DPH emission. The decrease in DPH life-time is due to the proximity of cytochrome c and the physiological cytochrome c:phospholipid ratio.

Three-dimensional Diffusion of Cytochrome c at the Mitochondrial Inner Membrane Surface—The RET from DPH incorporated into isolated mitochondrial inner membranes to cytochrome c (cytochrome c concentration, 2 μM; cytochrome c:phospholipid ratio, 1:83) was monitored at various ionic strengths (Fig. 1). For this ratio, the RET decreased, and the average distance of cytochrome c from the inner membrane increased with increasing ionic strength.

In intact mitochondria, the concentration of cytochrome c is greater than 100 μM depending on the mitochondrial configuration (23–25). However, due to the high absorbance of cytochrome c in the 410 nm region, the use of 100 or 700 μM exogenous cytochrome c with isolated inner membranes is not experimentally feasible for RET measurements. Therefore, various cytochrome c:phospholipid ratios were used with inner membranes for RET measurements in order to relate measurements on isolated inner membranes to the intact mitochondrion which contains a physiologically high cytochrome c concentration. A higher cytochrome c:phospholipid ratio resulted in a higher RET at each ionic strength (Fig. 2); and when these data were normalized such that RET at zero ionic strength was 100% for each cytochrome c:phospholipid ratio, the relative RET was identical for each ionic strength. These data show that the degree of RET is independent of the cytochrome c:phospholipid ratio and consequently, the cytochrome c concentration irrespective of ionic strength. Thus, by extrapolation, these results for the isolated inner membrane indicate that at physiological ionic strength, physiological concentrations of cytochrome c, and the physiological cytochrome c:phospholipid ratio as found in the intact mitochondrion, little, if any, cytochrome c is bound to the inner membrane.

### Table I

| Ionic strength | Cytochrome c added | Fluorometric measurement of % FITC-cytochrome c bound | Direct binding measurement of % cytochrome c bound | Bound cytochrome c oxidase | Diffusion coefficient of cytochrome c | %
|---------------|-------------------|------------------------------------------------------|-----------------------------------------------------|---------------------------|--------------------------------------|---
| mM           | μM                |                                                      |                                                     |                           |                                      |---
| 0.3          | 2                 | 95                                                   | 95                                                  | 4.0                       | 5.0 × 10⁻¹¹                         | 79 |
|              | 10                |                                                      |                                                     |                           | 5.1 × 10⁻¹¹                         | 90 |
| 23           | 3                 | 95                                                   | 87.5                                                | 36.8                      | 5.3 × 10⁻¹¹                         | 96 |
|              | 10                |                                                      |                                                     |                           | 2.6 × 10⁻¹⁰                         | 96 |
| 56           | 18                | 15                                                   | 17                                                  | 7.2                       | 3.1 × 10⁻¹⁰                         | 93 |
|              | 20                |                                                      |                                                     |                           | 2.2 × 10⁻¹⁰                         | 94 |

*Native or FITC-cytochrome c.

*Based on the averages of the third and fourth columns.

*Percent fluorescence recovery.

### Table II

| Medium                  | Phase | Modulation | τ     |
|-------------------------|-------|------------|-------|
| 150 mM KCl, no cytochrome c | ns    | ns         | 8.74  |
| No KCl, no cytochrome c  | 8.72  | 8.66       |       |
| 150 mM KCl + 2 μM cytochrome c | 8.57  | 8.65       |       |
| No KCl + 2 μM cytochrome c | 7.22  | 8.00       |       |

Percentage of fluorescence Recovery

### Figure 1

Proximity of cytochrome c to the inner membrane as a function of ionic strength. Percent RET (■) and average distance (□) was measured for mitochondrial inner membrane (0.5 mg/ml protein) labeled with DPH incubated in various concentrations of KCl/Hₐₙ₀ medium in the presence of 2 μM cytochrome c. Medium, percent RET, and distance calculations are described under "Experimental Procedures."
Cytosine three-dimensional.

By cytochrome the accompanying article (46) that electron transfer mediated therefore, 10% of the cytochrome incubated with various concentrations of physiological ionic strength, i.e. when cytochrome near the membrane surface in three dimensions. Direct binding assays revealed that above approximately 100 mM ionic strength, virtually no cytochrome molecules were bound to the membrane surface (Fig. 3). Again, the equilibrium between the cytochrome c molecules at the surface of the inner membrane and those in the aqueous medium shifts toward free cytochrome c molecules in the aqueous medium, resulting in three-dimensional diffusion, as the ionic strength is raised to physiological.

Proximity of Cytosine c to the Inner Membrane—RET has been utilized as a "spectroscopic ruler" to measure the distance between two chromophores up to 100 Å (16). Moreover, RET has been used to determine changes in this distance due to changes in the interaction between the chromophores (33). We found that the RET from DPH incorporated into the mitochondrial inner membrane to cytochrome c decreases sharply as the ionic strength is raised; and at 50 mM ionic strength, the RET is only 18% of that at zero ionic strength. This decrease in RET as ionic strength is increased up to 150 mM is independent of different cytochrome c:cytochrome oxidase ratios for inner membranes. At physiological (100-150 mM) ionic strength, little, if any, cytochrome c molecules, regardless of cytochrome c concentration, are within the RET distance of 100 Å. These RET data, combined with our cytochrome c direct binding assay data, reveal that cytochrome c molecules diffuse in three dimensions, colliding randomly with the inner membrane surface at physiological ionic strength.

In the intact, whole mitochondrion, the outer membrane is impermeable to cytochrome c (34) and represents a boundary, confining the three-dimensional diffusion of cytochrome c to the intermembrane space. The maximum concentration of cytochrome c in the intermembrane space is approximately 700 μM (23-25). Even at this high concentration, cytochrome...
Diffusion of Cytochrome c

5245

c physically occupies only 0.6\% (v/v) of the intermembrane space. Three other intermembrane proteins have been identified at low concentrations in rat liver mitochondria; adenylate kinase, nucleoside diphosphokinase, and nucleoside monophosphokinase (9, 13). Considering these data, cytochrome c should be able to diffuse freely in the intermembrane space irrespective of its concentration or the presence of these proteins. The question of the physiological ionic strength of the intermembrane space of the intact mitochondrion is also important, but not usually considered. Since the outer membrane is freely permeable to most ions and low molecular weight metabolites, the ionic strength of the intermembrane space is most likely equal to that of the cytoplasm, i.e. 100-150 mM (22). Since with inner membranes, at ionic strength >90 mM, most of the cytochrome c, regardless of concentration, is outside the maximal RET distance of 100 Å, the mode of cytochrome c diffusion should be three-dimensional in the intact mitochondrial at physiological, 100-150 mM ionic strength.

Rate of Three-dimensional Diffusion of Cytochrome c—The three-dimensional diffusion of cytochrome c in the mitochondrial intermembrane space raises the question of its role in electron transfer, e.g. is the rate of three-dimensional diffusion rate-limiting? The initial step to answer this question is to determine the rate of three-dimensional diffusion of cytochrome c at physiological ionic strength and then to calculate the frequency of its collisions with its redox partners. The collision frequency then can be compared with the electron transfer frequency. This comparison will be presented in the accompanying article (46).

The three-dimensional diffusion coefficient of cytochrome c in water, calculated from the Stokes-Einstein equation, is 1.5 × 10^-9 cm^2/s. This diffusion coefficient compares closely to that of lysozyme, which is a small soluble protein (M, 14,400) similar to cytochrome c (M, 12,400). Studies on the three-dimensional diffusion of lysozyme, using interferometry, show that at low ionic strength and infinite dilution, its diffusion coefficient is 5.8 × 10^-10 cm^2/s; at 700 µM concentration, it is 6.2 × 10^-10 cm^2/s; at ionic strengths between 50 and 150 mM, it is 1.4 × 10^-10 cm^2/s (35). Using FRAP, similar low 10^-10 cm^2/s three-dimensional diffusion coefficients were obtained for lysozyme and other soluble proteins of similar size in phosphate-buffered saline, the ionic strength of which is approximately 150 mM (36). Thus, the lowest three-dimensional diffusion coefficient of cytochrome c at 150 mM ionic strength and at 700 µM concentration is approximately 1.0 × 10^-10 cm^2/s.

Collision Frequency of Cytochrome c with Its Redox Partners—We have previously measured the lateral diffusion of mitochondrial inner membrane redox components at ionic strengths lower than physiological and have calculated the bimolecular diffusion-controlled collision frequencies between specific redox partners from these measurements using the Hardt equation (37) for a homogenous diffusion system comprised of two-dimensionally diffusing redox partners (7). It should be pointed out that the Hardt equation for two dimensions assumes that all colliding reaction partners are inert, hard spheres confined to the membrane, but does not consider change in affinity of any reaction partner for the membrane surface at different ionic strengths. These assumptions are reasonable, and our previous calculations are adequate for the collision frequencies of cytochrome c at the lower ionic strengths where diffusion is essentially two-dimensional.

Since we have now determined the modes and rates of cytochrome c diffusion for ionic strengths from 0 to 150 mM, we can calculate the bimolecular diffusion-controlled collision frequencies of cytochrome c diffusing in two and/or three dimensions with its two-dimensionally diffusing redox partners, i.e. a heterogenous diffusion system, using the treatment of Astumian and Chock (38). This treatment also includes the affinities of cytochrome c for the inner membrane which apply at different ionic strengths. Accordingly, we can describe three collisional modes for cytochrome c, diffusing in three dimensions, with its membrane-restricted redox partners, colliding laterally. 1) Some cytochrome c molecules collide directly with their redox partner molecules; 2) some cytochrome c molecules collide nonspecifically with the membrane and remain adsorbed long enough to diffuse laterally or pseudo-laterally to collide with their redox partner molecules; 3) some cytochrome c molecules, without collision with their redox partner molecules, dissociate from the membrane surface such that the location of a subsequent collision is approximately independent of their previous locations on the membrane. The relative contribution of each of these paths can be calculated by a branching method for the membrane surface dynamics of these paths.

The probability that a collision between cytochrome c and a redox partner can occur depends upon various factors: the fraction of membrane surface covered by the membrane-restricted redox partner, the rates of lateral and three-dimensional diffusion of cytochrome c, the rates of lateral diffusion of the redox partners, the mean occupancy time of cytochrome c on the membrane, the rate of the nonspecific dissociation constant of cytochrome c with the membrane, the radius of the membrane, the concentration of cytochrome c and redox partner; and the radius of the reactive area of cytochrome c and redox partner. Collectively, these factors contribute to the probability of one of the collisional modes being more favorable for collision.

Specifically, the probably that a collision between a molecule of cytochrome c and a membrane-restricted redox partner molecule will occur is dependent on both r and k_d (Table III). r is defined as the mean occupancy time for cytochrome c on the membrane to collide with a redox partner (Table III, sixth column) and is derived from the experimental diffusion coefficients of cytochrome c and its redox partner (fourth column), as well as from the effective quantity of the redox partner (second column) and other parameters (see Table III, Footnote c). It has been shown that the k_d of cytochrome c with various membrane types increases with increasing ionic strength (41-45). Compiling the data from these sources, we have selected the k_d of cytochrome c for the inner membrane as 1 at zero mM, 1 × 10^7 at 50 mM, and 1 × 10^7 at 150 mM ionic strength.2 At low ionic strength (Table III, third column), τ values are long (sixth column), and k_d values are low (seventh column); thus, cytochrome c tends to remain adsorbed to the membrane, and the probability of collision with either of its redox partners is maximum (eighth column). At high ionic strength, τ values are short, and k_d values are high; thus, cytochrome c tends to dissociate from the membrane, and the probability of collision with either of its redox partners decreases (eighth column). Using the experimentally determined τ values and the k_d values selected above, the values for the collision frequencies between cytochrome c and either of its redox partners at the three ionic strengths are obtained (ninth column).

2 The dissociation rate constant (k_d) of cytochrome c for inner membranes at various ionic strengths was calculated from the equilibrium association and dissociation constants as well as from the association rates of cytochrome c for phospholipid vesicle membranes or for solubilized cytochrome oxidase reported in the literature cited above.
by the method of Astumian and Chock we have calculated the collision frequencies between the reaction coefficients data show that any reduced cytochrome bcl-oxidized cytochrome oxidase (oxidized) 150 1.0 cytochrome bc, 919 5246 5

Cytochrome bcl (oxidized) 150 1.0

Cytochrome bcl (reduced) 1,880 0 4.3 \times 10^{-10} Lateral 3.1 \times 10^{-3} 1 1.0 8.7 \times 10^{19}

Cytochrome oxidase (oxidized) 10,646 50 2.3 \times 10^{-10} Pseudo-lateral 5.9 \times 10^{-4} 1 \times 10^4 1.0 8.7 \times 10^{23}

Cytochrome oxidase (reduced) 150 1.0 \times 10^{-6} Three-dimensional 1.4 \times 10^{-6} 1 \times 10^6 0.91 7.5 \times 10^{21}

*From the data of Klingenberg and Kroger (39), state 3U (uncoupled), calculated as described by Gupta et al. (7).

A lateral diffusion coefficient of cytochrome c from Table I and its membrane-restricted redox partners (Ref. 7; D for cytochrome bc = 4.3 \times 10^{-19} cm^2/s, and D for cytochrome oxidase = 3.7 \times 10^{-19} cm^2/s) are added (40) to calculate \( r \). At zero ionic strength, the diffusion is treated as ideally lateral; at 50 mM ionic strength, the diffusion is pseudo-lateral. However, it is treated as an effective, two-dimensional, lateral diffusion (40). At 150 mM ionic strength, the diffusion of cytochrome c is essentially three-dimensional with D = 1.0 \times 10^{-15} cm^2/s as determined in the text. This three-dimensional diffusion value of D represents the theoretical upper limit for lateral diffusion and therefore is used to calculate the highest collision frequencies possible.

Mean occupancy time for cytochrome c on the membrane, \( r = (1.1) (r_{\text{rel}}/N_d/D_{\text{av}}) \ln (1.2) (r_{\text{rel}}/N_d/D_{\text{av}}) \), where \( D_{\text{av}} \) = lateral diffusion of cytochrome c and its redox partners as described in Footnote b, \( r_{\text{rel}} = \text{average radius of the mitochondrial inner membrane (7.5 \times 10^{-10} \text{ cm}^2) \), N_d = \text{number of membrane-restricted reactive redox partners as in the second column, and } r_d = 2.5 \times 10^{-7} \text{ cm for both cytochrome bc and cytochrome oxidase. Radii are from Gupta et al. (7).}

Membrane surface affinity or nonspecific dissociation rate constant of cytochrome c.

Probability of collision of cytochrome c with either of its redox partners via three collisional modes as defined in text. \( p = (\alpha + \beta(1-\alpha))/(1-((1-\alpha)(1-\beta))\gamma) \), where \( \alpha = N_d r_d/(4r_{\text{rel}}^2) \), \( \gamma = r_{\text{rel}}/(r_{\text{rel}} + r_d) \), and \( \beta = 1/(1 + K_d \cdot r) \).

Diffusion-controlled collision frequency. \( \phi = [r_d D_{\text{av}} (1-\chi)]/[r_{\text{rel}} (1-\chi)] + [4 r_d D_{\text{av}} (1-\alpha)(\gamma-1)/N_d (1-\chi)] (k_d + r) [A] N_d \), where \( D_{\text{av}} = \text{three-dimensional diffusion of cytochrome c (D = 1.0 \times 10^{-14} cm^2/s, as determined in text, } X = ((1-\alpha)(1-\beta))\gamma, \text{ and A = concentration of three-dimensionally diffusing cytochrome c (A = 5.34 \times 10^{14} oxidized cytochrome c molecules and A = 6.6 \times 10^{23} reduced cytochrome c molecules).}

To understand better the effect of \( k_d \) on the collision frequency as well as to verify the appropriateness of the \( k_d \) values selected to determine the collision frequencies in Table III, we have calculated the collision frequencies between the reduced cytochrome bc, oxidized cytochrome c redox pair (Fig. 4) and the reduced cytochrome c-oxidized cytochrome oxidase redox pair (not shown) over a range of \( k_d \) values for each of the three diffusion rates in Table III (fourth column). The data show that any \( k_d \) below 1 \times 10^6 results in a maximum collision frequency, whereas any \( k_d \) above 1 \times 10^8 results in a minimum collision frequency regardless of diffusion rate. This underscores the significance of \( k_d \) in addition to the diffusion rate in determining diffusion-based collision frequencies. At higher ionic strengths, the higher \( k_d \) values and shorter \( r \) values as well as the RET and binding data indicate that the lower collision frequencies are due to a lower concentration (affinity) of cytochrome c near the surface of the membrane. These relationships will be discussed further in the accompanying article (46) with respect to electron transport. These results also show the appropriateness of the three \( k_d \) values (1, 1 \times 10^7, and 1 \times 10^9) selected to calculate the collision frequencies in Table III.

Our data reveal the significance of diffusion and concentration (affinity) of cytochrome c near the surface of the inner membrane in the control of its collision frequency with its redox partners at any ionic strength. Considering our experimental finding that cytochrome c diffuses in three dimensions at higher and physiological ionic strengths, the calculations reported in this study represent a more definitive approach to determine the collision frequencies for cytochrome c with its redox partners. Comparison of the theoretical collision frequency with the experimental rate of electron transport, reported in the accompanying article (46), will show the significance of the three-dimensional diffusion-based collision frequencies of cytochrome c with its redox partners in mediating electron transport in the isolated inner membrane as well as in the intact, whole mitochondrion at physiological ionic strength.

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