Mobility of Fluorescent Derivatives of Cytochrome c in Mitochondria

JANE M. VANDERKOOI, GRZEGORZ MANIARA, and MARIA ERECINSKA
Department of Biochemistry and Biophysics and Department of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia 19104

Abstract
Motion of cytochrome c bound to giant (2–10-μm diam) mitochondria isolated from the waterbug Lethocerus indicus was examined using the technique of fluorescence recovery after photobleaching. Fluorescent cytochrome c was exchanged for native cytochrome c through partly damaged outer membrane. Recovery profiles were not statistically different when the fluorescence from iron-free cytochrome c or fluorescein-labeled cytochrome c was used and were essentially the same in the presence or absence of an uncoupler. In the presence of excess porphyrin cytochrome c, the apparent diffusion coefficient was 6 × 10⁻¹¹ cm²/s in 0.3 M sucrose-mannitol-EDTA and 3 × 10⁻¹⁰ cm²/s in 0.10 M KCl/0.10 M sucrose. At concentrations of porphyrin cytochrome c that are stoichiometric with cytochrome c oxidase and for mitochondria in which excess cytochrome c was washed away, two components were observed in the recovery profile. The diffusion coefficient of the fast component was 1 × 10⁻¹⁰ cm²/s. The second component showed no recovery during the time scale of measurement (D < 10⁻¹² cm²/s). We speculate on the origin of the immobile fraction.

Cytochrome c is a small extrinsic membrane protein that transfers electrons between two large intrinsic complexes, cytochrome c oxidase and cytochrome bc₁ (for review, see Dixit and Vanderkooi [5]). Free diffusion of cytochrome c, either laterally on the membrane or in the intermembrane space, could accomplish this shuttle. Measurement of the lateral diffusion of cytochrome c by fluorescence recovery after photobleaching (FRAP)¹ in two laboratories has suggested that cytochrome c is freely diffusible (11, 14), but the interpretation of the data is a matter of controversy. The first group suggested that the rates of cytochrome c diffusion are compatible with its role as a mobile electron carrier (11) whereas the second group interpreted their results in terms of a dynamic aggregate between cytochrome c and its redox partners (14). However, cytochrome c that is covalently attached to the membrane can transfer electrons (7), and cross-linked cytochrome c in glutaraldehyde-treated mitochondria is also active (27). In addition, the existence of high-affinity binding sites (K < 10⁻⁵) on both the oxidase (8) and reductase (3) argues against the diffusion of free cytochrome c as a necessary prerequisite for electron transfer.

In this paper we reinvestigate the question of the mobility of cytochrome c bound to mitochondria using the FRAP technique. Our experiments differ from other work in the following aspects: (a) Giant mitochondria, isolated from the waterbug, Lethocerus indicus, were used; these mitochondria are naturally large without preliminary manipulations of animals that involve toxic treatment with drugs. (b) The preparation contained a population that were somewhat swollen and had the outer membrane only partially attached. (c) The iron-free derivative ("porphyrin" cytochrome c) was used; the intrinsic porphyrin of this derivative has high fluorescence quantum yield which permits examination of low concentrations. It has previously been shown that porphyrin cytochrome c is a competitive inhibitor of the native protein (28) with an inhibitory constant about the same as the binding constant for native cytochrome (30, 31); since the inhibition is competitive, it can be assumed that the inactive derivative binds at the same site as the native cytochrome c. (d) Concentration of added porphyrin cytochrome c was varied and "washed" mitochondria with tightly bound cytochrome c were examined.

Materials and Methods
Type III and type VI horse heart cytochrome c, fluorescein isothiocyanate, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Iron-free

Abbreviations used in this paper: Dil, 1,1'-dioctadecyl-3,3',3',3'-tetramethyl-indocarbocyanine perchlorate; FRAP, fluorescence recovery after photobleaching; MSE, 0.225 M mannitol, 0.075 M sucrose, and 0.002 M EDTA.
porphyrin cytochrome c was prepared and purified on Sephadex and CM-cellulose as previously described (28). Further purification was accomplished by chromatography on Sephadex G-50-120 columns (1 x 20 cm). The protein fraction contained no dimerized form of protein. Dil [1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate] was obtained from Molecular Probes, Inc. (Junction City, OR). Fluorescein-labeled cytochrome c was prepared according to Hochman et al. (14). Other chemicals were the highest purity commercially available. Water was deionized and distilled.

Fluorescence photobleaching: The fluorescence photobleaching apparatus was designed along conventional lines (15). A 2-W Argon-ion laser from Coherent Inc. (Palo Alto, CA) serves as the light source. The line at 514.5 nm was used. The microscope is a Leitz Ortholux II equipped for ploem entrance and a biconvex lens (f = 5 cm) focuses the beam to a waist on the cytochrome c plane. An EM-9865 B (EMI-Gencom, Inc., Plainview, NY) photomultiplier is mounted above the vertical output of the trinocular head and a multiplier is mounted above the vertical output of the trinocular head and a counter/accumulator (C-10) were all purchased from EMI Gencom, Inc. The counter/accumulator (C-10) were all purchased from EMI Gencom, Inc. The BCD output from the C-10 counter is interfaced to the Apple II microcomputer which also drives a second illuminating beam from the bleaching beam is separated by an EM-1200, 1985

**Preparation of Mitochondria:** Giant mitochondria were isolated from flight muscle of the waterbug, *Lethocerus indicus*. The insects were obtained from Thailand. Flight muscle tissue (0.15-0.25 g) was gently homogenized in 15 ml of isolation medium containing 0.15 M KCl, 0.0015 M EGTA, and 50 mM Tricine (pH 7.1). The homogenate was filtered through four layers of cheesecloth which was wet with isolation medium. The filtrate was centrifuged at 80 g for 5 min. The pellet was discarded and the supernatant was centrifuged at 3,600 g for 5 min. The pellet was resuspended and then centrifuged twice in the isolation medium and finally resuspended in 2 ml of buffer containing 0.225 M mannitol, 0.075 M sucrose, and 0.002 M EDTA (MSE), pH 7.1. In Fig. 3 the resuspension medium included KCl as indicated in the legends. All procedures were carried out in a water-ice bath.

**Spectral Studies:** Mitochondria (~2 mg protein/ml) were suspended in 50 mM phosphate buffer, pH 7.4, containing 1% sodium deoxycholate, and their spectral properties were measured in an Amino DW2A double beam/split beam spectrophotometer (American Instrument Co., Inc., Silver Springs, MD). The oxidized base-line was obtained after addition of 1 mM K-ferricyanide to both the sample and the reference cuvettes whereas the reduced spectrum was recorded after addition of sodium dithionite to the sample cuvette. The difference between the reduced (experimental) and oxidized (reference) samples was recorded.

**Labeling Mitochondria with Fluorescent Derivatives of Cytochrome C:** Mitochondria (1.5-2 mg protein/ml) were incubated with the cytochrome c derivatives for 15 min in 0.3 osmol solutions at room temperature. In cases where KCl was increased, we maintained the osmolarity by decreasing the amounts of MSE. These suspensions were used for the "unwashed" samples in the FRAP measurements. The "washed" samples excess of the derivatives of cytochrome c was removed by centrifugation for 5 min at 3,600 g. The pellet was resuspended in the same medium that was used for incubation.

**Results**

**Size and Oxidative and Spectral Properties of Flight Muscle Mitochondria**

Table I shows the rates of oxygen uptake of flight muscle mitochondria oxidizing three different substrates with (state 3) and without (state 4) ADP. It can be seen that the highest respiratory activity was attained with succinate whereas malate + glutamate was oxidized at a rate four- to sixfold slower. α-Glycerophosphate was a poorer substrate than succinate but better than malate (+ glutamate). These substrate specificities are characteristic of muscle mitochondria. The respiratory control ratios for the three substrates were 1.7, 1.8, and 2.7, respectively. The ADP/O ratios were 1.6 for succinate, 1.7 for α-glycerophosphate, and 2.8 for malate (+ glutamate). In all the experiments described above, the assay medium contained

| Substrate               | −ADP | +ADP
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>75 ± 5</td>
<td>125 ± 7</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>24 ± 3</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>Malate (+) glutamate</td>
<td>12 ± 2</td>
<td>32 ± 3</td>
</tr>
</tbody>
</table>

The incubation medium contained: 0.225 M mannitol, 0.075 M sucrose, 0.002 M EDTA, 0.005 M KCl, 0.005 M NaP, 0.2% BSA, 0.01 M substrate, and 0.5-1 mg of mitochondrial protein (pH = 7.4). ADP, where indicated, was added at 0.3-0.6 μM. Values are means ± SD for three experiments.
0.2% BSA. In the absence of albumin, respiration in state 4 was increased, with no change in state 3, which resulted in smaller respiratory control ratios.

The cytochrome content of the mitochondria was estimated by absorption spectroscopy. The overall properties of the spectra were similar to those of other mitochondrial preparations: there were prominent peaks at 551, 562, and 605 nm characteristic of cytochrome c, b, and a, respectively. The concentration of cytochrome aa₃ was 0.85 nmol/mg of protein and the relative ratios of the cytochromes were within the range of values measured for mitochondria from other sources.

The flight muscle mitochondria suspended in MSE buffer showed oxygen uptake with 10 mM ascorbate that was greatly enhanced by the addition of 1 μM cytochrome c. Since the outer mitochondrial membrane of intact organelles is essentially impermeable to exogenous cytochrome c (21, 32), this observation means that the outer membrane of the giant mitochondria was partially damaged and permeable to added cytochrome c.

To further characterize the preparation, we examined the mitochondria under a light microscope (Fig. 1). In isotonic MSE medium the size of the diameter varied from 2 to 10 μm and occasionally was up to 20 μm (Fig. 2A). When the medium was diluted by one third with distilled water we observed that the small (i.e., 2 μm) mitochondria expanded suddenly to ~5–20 μm and then did not change in size. By using dark-field optics, we saw that the outer membrane was disrupted but still attached to the inner membrane (Fig. 1B). The mitochondria suspended in the diluted MSE fragmented within 30–90 min and thus proved unsatisfactory for routine FRAP experiments. Therefore, in all our measurements, we used only those organelles that were at least 5 μm-diam and usually ≥10 μm. It was assumed, in agreement with the accessibility of mitochondria to exogenous cytochrome c described above, that the population of largest mitochondria which was isolated and maintained in the MSE buffer represented that of swollen organelles.
FRAP Profiles for Porphyrin Cytochrome c

The FRAP profiles for porphyrin cytochrome c in the presence of the giant mitochondria were examined as a function of porphyrin cytochrome c concentration. At high concentrations (20–80 μM) in which the stoichiometry of added porphyrin cytochrome c to cytochrome c oxidase was greater than 5:1, the recovery was 100% complete and the mean diffusion coefficient was 6.2 × 10⁻¹⁰ cm²/s in MSE, independent of porphyrin cytochrome c concentration. At low concentrations of porphyrin cytochrome c, the apparent diffusion coefficient appeared to be somewhat higher than for high concentrations and recovery was only ~50% (Fig. 2). The diffusion coefficients and recovery percentages are summarized in Table II.

The recovery profiles for fluorescein-labeled cytochrome c were obtained in several experiments (data not shown). We found no difference between fluorescein-cytochrome c and porphyrin cytochrome c in the recovery at concentrations that were in excess of the oxidase (>5:1 cytochrome c to oxidase). The detection of fluorescence by our instrument was not sensitive enough to measure lower concentrations of the fluorescein derivative. In some experiments we added the uncoupler, 1799 (2,6-dihydroxy 1,1,1,7,7,7, hexafluoro-2,6-

![Figure 2](https://example.com/figure2.png)

**Figure 2** FRAP profiles as a function of added porphyrin cytochrome c. Mitochondria (2 mg protein/ml) ~2 μM in cytochrome c) in MSE were incubated for 15 min with the following concentrations of porphyrin cytochrome c: (1) 5 μM; (2) 10 μM; (3) 20 μM; (4) 50 μM; or (5) 80 μM. Arrow indicates the time when the bleaching beam was applied for 0.5 s. The points represent the experimental values and the solid lines are the computer best fit which gave these values for D: 2.2 × 10⁻¹⁰, 1.1 × 10⁻¹⁰, 3.4 × 10⁻¹¹, 2.2 × 10⁻¹¹, and 5.8 × 10⁻¹¹ cm²/s for 1–5, respectively, and for apparent recovery: 48, 51, 97, 94, and 99% for 1–5, respectively.

Despite the problems of stability of the mitochondria in hypotonic medium, we also attempted to examine the FRAP profiles of porphyrin cytochrome c in mitochondria in MSE that was diluted by one third with water. We saw no difference in the recovery profiles for either low (5 μM) or high concentrations (50 μM) per 2 mg of mitochondrial protein between these swollen mitochondria and those examined above (Fig. 2 and Table II).

Effect of KCl on Binding and FRAP Profiles of Porphyrin Cytochrome c to Mitochondria

KCl dislodges bound porphyrin cytochrome c from the membrane (Fig. 3). This is well known and is indicative of the ionic binding of cytochrome c with the membrane (5).

Recovery of fluorescence after bleaching was faster after addition of KCl in the presence of excess porphyrin cytochrome c (Table III). The diffusion coefficient in the absence of KCl was 6.2 × 10⁻¹⁰ cm²/s and 33 × 10⁻¹¹ cm²/s with 50 mM KCl (Table III). The effect of KCl is consistent with the off-rate that determines the apparent diffusion coefficients. This is discussed later.

In washed mitochondria the apparent diffusion coefficient was somewhat higher than in unwashed mitochondria at the same ionic strength (Table III). A higher diffusion coefficient was also observed at low porphyrin cytochrome c concentrations (Table II) in agreement with the results obtained on washed mitochondria. Analysis of variance revealed that there is no significant difference between different preparations of mitochondria.

**FRAP Profiles for Consecutive Bleaches**

In the experiments taken at low porphyrin cytochrome c concentrations, 100% recovery may not have been achieved because the sample was too extensively bleached. However, the observation that the percent recovery was nearly independent of the bleaching time and extent of the total bleach provides evidence that this is not a case. Further indication that the sample was not over-bleached was obtained from consecutive bleaches of the same spot. FRAP profiles for consecutive bleaches of washed mitochondria are shown in

---

**Table II**

<table>
<thead>
<tr>
<th>Concentration* μM</th>
<th>N</th>
<th>Recovery %</th>
<th>D cm²/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>5</td>
<td>97 ± 3</td>
<td>6.4 ± 1.5 × 10⁻¹¹</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>93 ± 3</td>
<td>7.1 ± 0.1 × 10⁻¹¹</td>
</tr>
<tr>
<td>40</td>
<td>2</td>
<td>99 ± 1</td>
<td>6.5 ± 1.6 × 10⁻¹¹</td>
</tr>
<tr>
<td>20</td>
<td>23</td>
<td>96 ± 2</td>
<td>6.2 ± 0.2 × 10⁻¹¹</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>57 ± 6</td>
<td>1.5 ± 1.3 × 10⁻¹⁰</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>55 ± 10</td>
<td>2.4 ± 1.6 × 10⁻¹⁰</td>
</tr>
</tbody>
</table>

| * Mitochondria were prepared as described in Materials and Methods and then labeled with porphyrin cytochrome c by incubation in the concentrations indicated in the table for 15 min at room temperature. FRAP measurements were done as described in the legend of Fig. 2.
| N Number of FRAP measurements taken.
| % Mean percent recovery ± SD.
| cm²/s Diffusion coefficient ± SD.

---

**bis[trifluoromethyl]-heptanone-4 bis[hexafluoroacetonyl] acetone; 10 μM). We could see no difference in the recovery curves in the presence and absence of uncoupler.**
Figure 3  Binding as a function of KCl. Mitochondria (1.8 mg/ml) were incubated with 20 μM porphyrin cytochrome c in MSE for 15 min, followed by centrifugation at 3,600 g for 5 min. The pellet was resuspended in KCl concentrations indicated in the figure and MSE to give 0.3 osmol. After a 15-min incubation, the sample was centrifuged for 5 min at 3,600 g. The pellet was resuspended in the same medium, incubated for 15 min, and recentrifuged. The amounts of porphyrin cytochrome c in the pellet and the two supernatants were determined by the fluorescence intensity at 620 nm. The values for “free” in the figure indicate the sum of the two supernatants.

Figure 4. We can see that on the first bleach the recovery was 50% but by the third bleach the recovery was 80% of the intensity obtained after recovery from the second bleach. This would indicate that the immobile phase did not recover on this time scale and the contribution of the “mobile” molecules of cytochrome c was relatively larger after the second and further bleaches. It also suggests that the “unrecovered” fluorescence cannot be accounted for by over-bleaching and that immobilization was not due to a laser effect which cross-linked a mobile fraction, since in these cases the percent recovery would be expected to be the same each time.

Lipid Mobility in Giant Mitochondria

The lipid probe Dil was also examined for lateral diffusion. We found that the recovery approached 100% (93 ± 19, n = 5), which is another indication that the sample was not too extensively bleached. The diffusion coefficient was 2.0 ± 1.8 × 10⁻⁹ cm²/s. This value is somewhat less than that found in other membranes, but this could be due to the extraordinarily high protein content of mitochondria as compared with other membranes.

DISCUSSION

Mitochondria prepared from the waterbugs showed respiratory control and normal concentrations of the cytochromes. The preparation as isolated contained organelles that were swollen and whose outer membrane was partially damaged: ascorbate oxidation was increased by the addition of cytochrome c, which normally does not penetrate intact outer

Table III

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Recovery</th>
<th>D⁺</th>
<th>Mean D⁺</th>
<th>ANOV,F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed, no KCl</td>
<td>4</td>
<td>93 ± 4</td>
<td>5.8 ± 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>96 ± 3</td>
<td>5.9 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75 ± 20</td>
<td>8.3 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>98 ± 6</td>
<td>5.5 ± 2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>98 ± 5</td>
<td>5.9 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>96 ± 2</td>
<td>8.4 ± 1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed, no KCl</td>
<td>6</td>
<td>66 ± 10</td>
<td>19 ± 9</td>
<td>f₁₀,₀₀₁,₂,₂,₁₅</td>
<td>2.81</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>57 ± 13</td>
<td>20 ± 5</td>
<td>16 ± 4</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>60 ± 8</td>
<td>8.4 ± 0.6</td>
<td>4.38</td>
<td></td>
</tr>
<tr>
<td>Unwashed, 25 mM KCl</td>
<td>4</td>
<td>85 ± 18</td>
<td>17 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>84 ± 8</td>
<td>20 ± 8</td>
<td>16 ± 4</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>99 ± 2</td>
<td>15 ± 4</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Washed, 25 mM KCl</td>
<td>3</td>
<td>46 ± 6</td>
<td>37 ± 3</td>
<td>f₁₀,₀₀₁,₂,₆</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>64 ± 7</td>
<td>28 ± 13</td>
<td>25 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>49 ± 7</td>
<td>28 ± 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>58 ± 12</td>
<td>19 ± 11</td>
<td>0.51</td>
<td>3.34</td>
</tr>
<tr>
<td>Unwashed, 50 mM KCl</td>
<td>3</td>
<td>98 ± 2</td>
<td>32 ± 6</td>
<td>33 ± 5</td>
<td>f₁₀,₀₀₁,₄</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>96 ± 2</td>
<td>36 ± 12</td>
<td></td>
<td>7.71</td>
</tr>
<tr>
<td>Unwashed, 100 mM KCl</td>
<td>3</td>
<td>98 ± 1</td>
<td>36 ± 17</td>
<td>32 ± 10</td>
<td>f₁₀,₀₀₁,₄</td>
</tr>
<tr>
<td>Washed, uncoupler</td>
<td>3</td>
<td>96 ± 3</td>
<td>17 ± 3</td>
<td></td>
<td>7.71</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>69 ± 8</td>
<td>27 ± 10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mitochondria were incubated with 20 μM porphyrin cytochrome c as described in Materials and Methods except in the case indicated by ***, where the concentration was 40 μM.
† Number of measurements on different mitochondria from the same mitochondrial preparation.
* Mean percent recovery ± SD.
* Diffusion coefficient (D) ± SD.
** Mean D for all experiments ± SD.
*** Analysis of variance (ANOV); F is calculated as described (35).
**** F values from tabulated values for α = 0.05 (35).
membrane. The fragility of the large mitochondria suspended in the hypotonic media did not allow us to use sucrose gradient centrifugation for purification of the mitoplasts. However, we incubated the mitochondria with porphyrin cytochrome c for 15 min to allow for its penetration into the intermembrane space through the disrupted outer membrane and its exchange with bound, endogenous cytochrome c. Since porphyrin cytochrome c is a competitive inhibitor of native cytochrome c, we feel that under these conditions at least a part of porphyrin cytochrome c was bound at the physiological, high-affinity sites located on the outer surface of the inner mitochondrial membrane.

Under conditions of excess porphyrin cytochrome c, our results compare favorably with those obtained by others. Hochman et al. (14) found a diffusion coefficient of 1.6 x 10^{-10} cm²/s for a 10-fold excess of cytochrome c bound to mitochondria obtained from cuprizone-treated mice. Gupta et al. (11) report a value of 6 x 10^{-11} cm²/s for calcium-fused inner mitochondrial membranes at low ionic strength. These values are not substantially different from the figure of 6.2 x 10^{-11} cm²/s reported here and obtained under similar experimental conditions. It should be mentioned, that although the specific high-affinity binding sites for cytochrome c on the outer surface of the inner mitochondrial membrane should be preferentially filled in our studies, binding to the fragments of the outer membrane can neither be excluded nor reliably quantified. Moreover, in view of the lack of information on the distribution of the marker enzyme for the outer membranes in the preparation of previous investigators (11, 14), it is impossible to evaluate the extent of interference from this source of error in their studies. However, the agreement between our results and those of other authors would suggest that either the nature (i.e., localization) or the properties (i.e., protein, lipid, or both) of the binding sites for fluorescent derivatives of cytochrome c were similar in all three studies.

Gupta et al. (11) and we have observed that with increasing ionic strength there is an increase in the apparent diffusion rate for cytochrome c derivatives, when they are in excess. The effect of KCl at excess concentrations of porphyrin cytochrome c suggests that the apparent diffusion rate is really a reflection of the exchange between bound and free molecules. Further evidence for this suggestion is the finding that at high concentrations of porphyrin cytochrome c the apparent diffusion coefficient was independent of porphyrin cytochrome c concentration (Table II).

If at high concentrations of porphyrin cytochrome c (>20 μM) the FRAP profile reflects the exchange between free and bound cytochrome, and if the off-rate is solely determining the exchange rate between them, then the recovery profile should fit to a simple exponential (26). We compared curves simulated by Eq. 1 for diffusion and simulated by an exponential for rebinding with representative recovery curves. The shapes of the simulated curves were not sufficiently different to distinguish which better describes the experimental data and so the conclusion that the recovery curves reflect the rebinding of cytochrome c cannot be overruled. Therefore, the values for “diffusion” coefficient given in Tables II and III reflect a binding component and are not solely accounted for by diffusion.

Perhaps the most striking observation of this study is that at porphyrin cytochrome c concentrations that are about the same as native cytochrome c (i.e., 2:1 cytochrome c to cytochrome c oxidase), there is an immobilized fraction. It is reasonable to assume that at these low concentrations the high-affinity sites are predominantly filled. We suggest the following possibilities with respect to the origin of the immobilized fraction:

(a) Cytochrome c is bound with high affinity to cytochrome c oxidase and reductase and these components may be immobilized because they aggregate into large complexes.

(b) It is well known that certain plasma membrane proteins diffuse very slowly (or not at all) because of interaction with a supporting system. Interaction of plasma membrane proteins with the cytoskeleton or membrane skeleton in erythrocytes, for example, has been reported (4, 20). In addition, the glyocalyx has been suggested to restrict mobility of plasma proteins (33). In mitochondria, the outer membrane could in effect be an exoskeleton, providing support. Alternatively, inner mitochondrial membrane could also be equipped with some kind of “skeleton.” We may point out that nonrandom distribution of the mitochondrial components can be seen in early electron micrographs such as those shown by Fernandez-Moran et al. (9) and Smith (24). It can
be seen (9) that the knobs, associated with the ATPase, are not randomly distributed, but concentrated on the cristae foldings; these observations can be used to argue against free mobility of respiratory chain components.

(c) Cytochrome c may be mobile within a small domain, but then may hit a barrier. The barrier may arise from several sources. Although we were using swollen mitochondria, we cannot rule out that convolutions of the inner mitochondrial membrane may present a barrier. Other barriers, such as immobilized membrane proteins which would corral cytochrome c, are also possible.

The authors thank Dr. David Jameson for setting up the FRAP equipment and Mr. Brian Hill and Mr. Vincent Moy for their help in computer programming. They also thank Dr. David White and Dr. Yale Goldman for assistance in obtaining the waterbugs and for pointing out that these insects contain giant mitochondria.

This work was supported by National Institutes of Health grant HL 18708. A preliminary report of this work was presented to the Biophysical Society, February, 1984 (22).

Received for publication 5 January 1984, and in revised form 9 October 1984.

REFERENCES


