Mobility in the Mitochondrial Electron Transport Chain†

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ABSTRACT: The role of lateral diffusion in mitochondrial electron transport has been investigated by measuring the diffusion coefficients for lipid, cytochrome *c*, and cytochrome oxidase in membranes of giant mitoplasts from cuprizone-fed mice using the technique of fluorescence redistribution after photobleaching (FRAP). The diffusion coefficient of the phospholipid analogue *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine is dependent on the technique used to remove the outer mitochondrial membrane. A sonication technique yields mitoplasts with monophasic recovery of the lipid probe ($D = 6 \times 10^{-9}$ cm^2/s), while digitonin-treated mitochondria show biphasic recoveries ($D_1 = 5 \times 10^{-9}$ cm^2/s; $D_2 = 1 \times 10^{-9}$ cm^2/s). Digitonin appears to incorporate into mitoplasts, giving rise to decreased lipid mobility concomitant with increased rates of electron transfer from succinate to oxygen, in a manner reminiscent of the effects of cholesterol incorporation [Schneider, H., Lemasters, J. J., Hochli, M., & Hackenbrock, C. R. (1980) *J. Biol. Chem. 255*, 3748–3756]. FRAP measurements on tetramethylrhodamine cytochrome *c* modified at lysine-39 and on a mixture of active morpholinorhodamine derivatives of cytochrome *c* gave diffusion coefficients of (3.5–7) $\times 10^{-10}$ cm^2/s depending on the assay medium. With morpholinorhodamine-labeled antibodies purified on a cytochrome oxidase affinity column, the diffusion coefficient for cytochrome oxidase was determined to be 1.5×10^{-10} cm^2/s. The results are discussed in terms of a dynamic aggregate model in which an equilibrium exists between freely diffusing and associated electron-transfer components.

Membrane-associated biochemical reactions are generally thought to occur by mechanisms that require lateral diffusion of membrane components. In some cases, it has been proposed that reactions in the membrane are dependent on random collisions (Liebman & Pugh, 1979, 1981; Yee & Liebman, 1978; Hanukoglu & Jefcoate, 1980), where diffusion itself is rate limiting, while in other cases the evidence suggests a mechanism of reversible aggregate formation (Haworth et al., 1982; Gut et al., 1983), for which diffusion is not rate limiting. One system in which random collisions have been suggested to be the dominant mode of interaction is the mitochondrial electron-transfer chain. This membrane-associated reaction sequence is particularly interesting because of its specialized role in conserving energy, a process whose molecular mechanism is yet to be established.

Early ideas concerning the structure of the mitochondrial inner membrane were influenced by the observation that it has an unusually high protein to lipid ratio (Colbeau et al., 1971), suggesting organized arrays of proteins. The possibility was considered that the electron transport chain might exist as a structural unit (Lehninger, 1959; Blair et al., 1963; Klingenberg, 1968), a concept that has been extended to include direct communication with the ATP-synthesizing machinery (Boyer, 1977; Chance, 1977, 1982). However, no direct chemical or conformational communication is required for an energy-transfer event involving a proton gradient as proposed by Mitchell (1961). In fact, experiments designed to investigate the degree of static organization among the inner mitochondrial membrane proteins have shown that despite the high protein content the large integral proteins are able to diffuse freely and independently over considerable distances (Hochli & Hackenbrock, 1976, 1978) and occupy only approximately half the total membrane area (Sowers & Hackenbrock, 1981). In addition, studies on the rotational mobility of cytochrome oxidase demonstrate that at least half the oxidase in the native mitochondrial membrane has free rotational mobility (Kawato et al., 1980). Schneider et al. (1980, 1982a,b) have also shown that artificially induced alterations in the distances between electron-transfer components are accompanied by changes in electron-transfer rates. When the distances between redox centers are decreased, the rate of electron transfer from succinate or NADH to oxygen is increased, while an increase in distance decreases the electron-transfer rates. These findings led to a proposal (Hackenbrock, 1981) that mitochondrial electron transfer occurs by a diffusion-mediated mechanism in which cytochrome *c* and ubiquinone diffuse much faster than the large integral protein complexes.

Cytochrome *c* has been suggested to communicate electrons between cytochromes bc_1 and aa_3 by rapid diffusion on the negatively charged membrane surface (Chance, 1974; Roberts & Hess, 1977; Margoliash & Bosshard, 1983; Froud & Ragan, 1984) with correct orientation of the mobile carrier maintained by its large dipole moment (Koppenol & Margoliash, 1982). This idea is supported by studies on the effects of chemical modification of cytochrome *c* on its reactivity with its redox partners, indicating that the same residues are involved in the binding of cytochrome *c* to both cytochrome aa_3 and cytochrome bc_1 (Brautigan et al., 1978a,b; Ferguson-Miller et al., 1978, 1979; Ahmed et al., 1978; Rieder & Bosshard, 1978a,b; Speck et al., 1979). Nevertheless, cytochrome *c* in rat liver mitochondria appears to have a rotational mobility similar to that of oxidase, implying their existence as a reasonably long-lived complex in the native membrane (Dixit et al., 1982). Moreover, when cytochrome *c* is cross-linked to mitochondrial inner membranes, significant rates of electron transfer are retained (Utsumi & Packer, 1967; Erecinska et al., 1975; Waring et al., 1980). This suggests that dissociation of cytochrome *c* from its redox partners may not be required for its activity.

Because of the small size and lipid nature of ubiquinone and its presence in excess to its redox partners (Vinogradov & King, 1979), it has generally been considered to function as a mobile electron carrier (Green, 1962). This concept is

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substantiated by a number of kinetic studies [Kroger & Klingenberg, 1973a,b; for a review, see Rich (1981, 1984)]. However, there is also evidence for direct interaction of cytochrome bc_1 (complex III) with complex I (Ragan & Heron, 1978; Heron et al., 1978a,b) and with succinate dehydrogenase (Yu et al., 1974, 1977), raising the possibility that electron transfer by ubiquinone can occur via direct association of the complexes as well as by free diffusion.

To address the ambiguities regarding the role of diffusion in mitochondrial electron transfer, and the existence and functional significance of physical associations among electron carriers, we have measured the rates of lateral diffusion of cytochrome *c*, cytochrome oxidase, and lipid in the membranes of giant mitochondria by fluorescence redistribution after photobleaching. A fluorescent derivative of cytochrome *c*, singly modified at residue 39 and having native activity, was used for these studies. The results provide the information necessary to quantitatively assess the feasibility of a completely random diffusion mechanism of electron transfer.[1]

Materials and Methods

Materials. Cuprizone [oxalic acid bis(cyclohexylidenehydrazide)] was purchased from G. Fredrick Smith Chemical (Columbus, OH) or from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized according to Bowman & Tedeschi (1983). CM-cellulose (Whatman, CM 52) was prepared by the manufacturer's suggested directions. Cytochrome *c* (Sigma type VI) was further purified according to Brautigan et al. (1978c). Prior to use in kinetic assays, cytochrome *c* was reduced with dithionite and gel filtered on Sephadex G-75 superfine (Pharmacia) in 25 mM tris(hydroxymethyl)-aminomethane (Tris)–cacodylate, pH 7.8, in order to remove polymerized cytochrome *c* (Brautigan et al., 1978c). Tetramethylrhodamine isothiocyanate was purchased from Polysciences, Warrington, PA, and morpholinorhodamine isothiocyanate was from Research Organics, Cleveland, OH.

Preparation of Giant Mitochondria. Seventeen to nineteen day old mice were fed a diet containing 3 g of cuprizone in 500 g of ground rodent chow and were supplied exclusively with deionized water. Six to twelve days after the initiation of the diet, giant mitochondria were prepared from the livers of these mice by using a modification of the procedure of Bowman & Tedeschi (1983). Three to five livers per 5 mL were minced, washed repeatedly in the isolation buffer [220 mM mannitol, 70 mM sucrose, and 2 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) adjusted with Tris to pH 7.4, containing 0.5 mg/mL bovine serum albumin (BSA)], and homogenized with a loose-fitting glass homogenizer. The homogenate was centrifuged at 120*g* for 1 min, and the supernatants were layered on top of 5 mL of 0.5 M sucrose and centrifuged for 10 min at 410*g*. The top layer which contained smaller mitochondria and other membranes was discarded, and the pellet was gently resuspended into the 0.5 M sucrose (lower) layer. The sample was diluted to 0.3 M sucrose with glass-distilled water and pelleted at 750*g* for 5 min. The pellet was resuspended in 5 mL of isolation buffer, layered on 0.5 M sucrose, and centrifuged at 240*g* for 3 min. The top layer was collected, and the giant mitochondria were pelleted at 720*g* for 5 min in 0.25 M surcrose. Unless indicated otherwise, the whole mitochondria were depleted of outer membrane according to the procedure of Sottocasa et al. (1967) as modified by Felgner et al. (1979), and the mitoplasts were pelleted at 3000*g* for 10 min.

The mitoplasts were resuspended in 250 mM mannitol and 50 mM Hepes, pH 7.2, and swollen by the gradual addition of 5 volumes of double-distilled water over 10 min to achieve the spherical configuration necessary to apply the edge-bleach technique for fluorescence recovery measurements (Koppel, 1979). In cases where cytochrome *c* diffusion was measured, whole mitochondria were depleted of cytochrome *c* by swelling the mitochondria in 20 mL of 10 mM Tris–phosphate, pH 7.5, followed by addition of 3 mL of 1 M KCl and pelleting prior to the removal of the outer membrane. The total phospholipid content of broken (freeze–thawed) mitoplasts was deduced by the procedure of Ames (1966). Lipids were extracted from the membranes according to the procedure of Awashthi et al. (1971) and chromatographed in two dimensions by using the solvent system of Parsons & Patton (1967) on Supelco Redicoat two-dimensional (2D) precoated thin-layer chromatography (TLC) plates which had been preextracted with methanol–methylene chloride (1:1). The lipid spots were visualized according to Thompson & Ferguson-Miller (1983).

The concentration of cytochrome oxidase in the mitochondria was determined from difference spectra (dithionite reduced minus ferricyanide oxidized) using $\Delta\epsilon_{605-630nm} = 24$ mM^{-1} (Von Jagow & Klingenberg, 1972). Mitochondrial protein concentrations were determined for the deoxycholate-solubilized membranes by using the biuret procedure.

Preparation and Characterization of Fluorescent Cytochrome c. Tetramethylrhodamine- and morpholinorhodamine-derivatized cytochromes *c* were prepared and purified as described previously (Hochman et al., 1982). The activity of derivatized and native cytochromes *c* with cytochrome *c* depleted rat liver mitoplasts was monitored in 25 mM Tris–cacodylate and 250 mM sucrose, pH 7.9, according to the method of Ferguson-Miller et al. (1976). The positions of the lysine modifications were determined from chymotryptic maps as described by Brautigan et al. (1978b).

Swollen, cytochrome *c* depleted mitoplasts were labeled with the fluorescent derivatives as reported previously (Hochman et al., 1982) and were pelleted at 750*g* for 5 min or were diluted 10-fold in 42 mM mannitol, 8 mM Hepes, pH 7.2, or 25 mM Tris–cacodylate, pH 7.9.

Preparation of Fluorescent Antibodies to Cytochrome aa_3. Cytochrome oxidase was prepared according to the method of Kuboyama et al. (1972) as modified by Suarez et al. (1984). The lauryl maltoside solubilized enzyme was dialyzed against phosphate-buffered saline (136 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM dibasic sodium phosphate, and 1.5 mM monobasic potassium phosphate, pH 7.2) and was mixed with an equal volume of adjuvant. Rabbits were injected subcutaneously at multiple sites on the back. The initial injection was with 4 mg of enzyme in Freund's complete adjuvant and was followed by repeated injections of 1 mg of enzyme in Freund's incomplete adjuvant at 2-week intervals. Blood samples were taken 7–10 days after each injection. Once a significant antibody response was observed, the frequency of injections was reduced to 2-month intervals.

The antibodies were purified on protein A–Sepharose (Sigma) according to Tucker et al. (1978) and dialyzed into 0.1 M sodium bicarbonate at pH 9.5. The dialysate was centrifuged at 10000*g* for 10 min in order to remove any precipitated protein. The antibody-containing supernatant was reacted with 0.15 mg of morpholinorhodamine isothiocyanate per milligram of antibody, and the reaction was allowed to proceed for 3 h. Free dye was separated from the labeled antibodies by gel filtration on Sephadex G-25 followed by

[1] Preliminary accounts of portions of this work have appeared (Hochman et al., 1983a,b).

overnight dialysis againt phosphate-buffered saline. The labeled antibody solution was applied to a 5-mL cytochrome oxidase affinity resin prepared from guanidine hydrochloride treated cytochrome oxidase according to Hackenbrock & Hammon (1975) using cyanogen bromide activated Sepharose 4B prepared by the methods of Cuatrecasas et al. (1968) or Kohn & Wilchek (1982). The column was washed with phosphate-buffered saline until no dye could be detected in the eluent, and the antibodies were eluted with 6 M guanidine hydrochloride, pH 5.6. The more tightly binding antibodies were pooled and dialyzed for 3 days against phosphate-buffered saline with repeated buffer changes. The fluorescent immunoglobulin G (IgG) was stored in phosphate-buffered saline at −20 °C.

Prior to labeling of the mitochondria, samples of the antibody preparations were dialyzed overnight against 42 mM mannitol and 8 mM Hepes, pH 7.2, and centrifuged at 10000*g* for 10 min to remove any IgG which may have denatured during storage. Swollen mitoplasts were incubated in antibody solution for 5 min, pelleted, and washed 3 times.

N-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) Labeling. Ten microliters of NBD-PE (Avanti, Birmingham, AL) (1 mg/mL in 100% ethanol) was added to a 1-mL suspension of mitochondria (0.5 mg of protein) in 42 mM mannitol and 8 mM Hepes, pH 7.2. The mitochondrial were incubated with the fluorescent probe for 5 min, washed repeatedly, and resuspended in the mannitol–Hepes buffer. Under these conditions, approximately half of the probe was incorporated into the membranes (≤5% of total lipid).

Digitonin-treated mitoplasts were prepared according to Schnaitman & Greenwalt (1968) in 220 mM mannitol, 70 mM sucrose, and 2 mM Hepes adjusted with Tris to pH 7.4, containing 0.5 mg/mL BSA.

Fluorescence Redistribution after Photobleaching. Fluorescence redistribution after photobleaching was performed by using the edge bleach technique developed by Koppel (Koppel, 1979; Koppel et al., 1980). The data were analyzed by using a normal-mode analysis for diffusion on spherical surfaces (Koppel et al., 1980). Rhodamine fluorescence was monitored with an incident wavelength of 514 nm and a combination of a Leitz TK 580 dichroic mirror and a K 570 barrier filter or a sharp-cut filter made from Corning glass 3482 (<1% transmittance below 544 nm; >90% transmittance above 567 nm). NBD fluoresence was monitored with an incident wavelength of 477 nm and a combination of a Leitz TK 510 dichroic mirror and a K 530 barrier filter.

Results and Discussion

Giant mitochondria for FRAP investigations were prepared from the livers of mice that had been fed a diet containing the copper chelator cuprizone (Bowman & Tedeschi, 1983). These mitochondria have levels of heme components and electron-transfer activities comparable to those found in normal rat liver mitochondria (Hochman et al., 1982) and exhibit respiratory control (Maloff, 1978; J. Hochman and S. Ferguson-Miller, unpublished results). Analysis of the total phosphorus content of isolated inner membranes reveals 15% by weight lipid made up of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin (data not shown). We conclude that these mitochondria with the exception of their unusually large size are structurally and functionally normal. Indeed, highly extended mitochondrial structures exist under a variety of natural conditions (Hoffman & Avers, 1973; Brandt et al., 1974; Maniara et al., 1984).

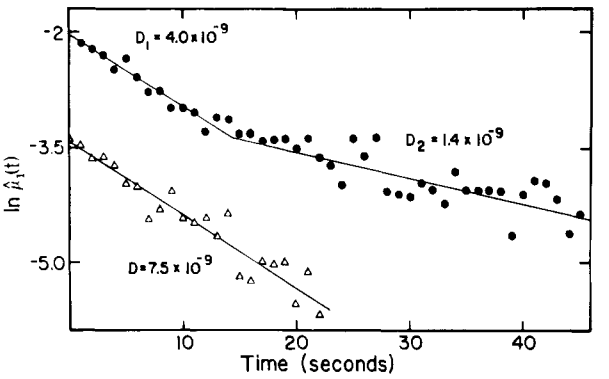

FIGURE 1: Comparison of the fluorescence recovery of NBD-phosphatidylethanolamine in mitoplasts prepared by digitonin treatment (●) and by mild sonication (△). Digitonin-treated mitoplasts were prepared by incubation of whole giant mitochondria at 50 mg of protein/mL with 0.12 mg of digitonin/mg of mitochondrial protein for 15 min. Scans were taken at 1-s intervals. The symbol $\hat{\mu}_1(t)$ reflects the proportion of fluorescent probe on the bleached edge of the mitochondrion at time t and is defined as the normalized first moment of the unbleached fluorophore concentration distribution (Koppel et al., 1980).

Lipid Diffusion. To investigate the behavior of lipids, we incorporated the fluorescent probe NBD-phosphatidylethanolamine into swollen inner mitochondrial membranes. We expect that the measurement of lipid diffusion will also provide an estimate of the lateral mobility of ubiquinone and serve to monitor the effects of membrane perturbing agents.

Under conditions where the fluorescent probe represented less than 5% of the total membrane lipid, diffusion rates were found to be dependent on the procedure used to remove the mitochondrial outer membrane. When a sonication technique was employed (Sottocasa et al., 1967), monophasic recovery of lipid was observed, yielding a diffusion coefficient of 6×10^{-9} cm^2/s (Figure 1). In contrast, mitoplasts prepared by treatment of mitochondria with digitonin (Schnaitman & Greenawalt, 1968) are found to have biphasic lipid recoveries in which the diffusion coefficient of the first phase ($D_1 = 5 \times 10^{-9}$ cm^2/s) is approximately the same as that for mitoplasts not exposed to digitonin while that for the second phase ($D_2 = 1 \times 10^{-9}$ cm^2/s) is significantly slower. The degree of biphasicity appears to be dependent on the amount of digitonin used, suggesting that the modified characteristics of lipid mobility are related to digitonin treatment. While digitonin inhibits lipid mobility, it accelerates rates of electron transfer in the succinate oxidase sequence of reactions (Figure 2). The activity of cytochrome oxidase itself, as measured with the artificial electron donors ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), is essentially unaffected by the digitonin treatment, indicating that accelerated electron transfer through the succinate oxidase chain was not due to a direct effect of digitonin on cytochrome oxidase.

Schneider et al. (1980, 1982a) previously reported a similar increase in mitochondrial electron-transfer activity when cholesterol was incorporated into inner membranes of rat liver mitochondria. Since digitonin is a steroid molecule solubilized by a hydrophilic polysaccharide chain, it is possible that it incorporates into the inner membranes of mitochondria and acts in a manner analogous to cholesterol, which appears to produce patching of the lipids and resultant aggregation of the proteins. Lipid extracts from digitonin-prepared mitoplasts reveal a spot corresponding to digitonin on TLC plates even after repeated washing of the membranes.

In the cholesterol incorporation experiments, Schneider et al. (1980, 1982a) accounted for the increased electron-transfer activities on the basis of decreased distances between the redox

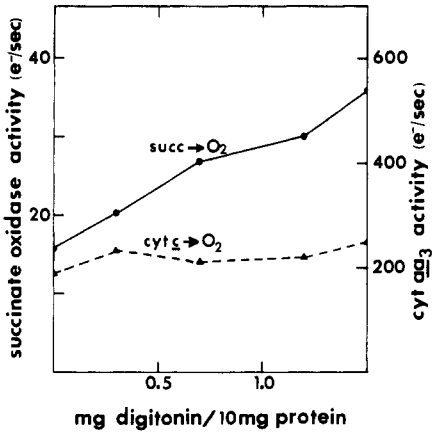

FIGURE 2: Effects of digitonin on succinate oxidase (●) and cytochrome oxidase (▲) activity. Whole giant mitochondria (50 mg/mL) were incubated with 0.03, 0.07, 0.12, or 0.15 mg of digitonin/mg of mitochondrial protein as detailed previously. In the case of zero digitonin, the outer membrane was removed by the sonication procedure described under Materials and Methods. Activities are expressed as electrons per second per cytochrome aa_3 and were obtained with mitoplasts at concentrations of 0.019 and 0.048 nmol of cyt aa_3/mL (for zero digitonin) and 0.099, 0.043, 0.085, and 0.033 nmol of cyt aa_3/mL for mitochondria treated with 0.03, 0.07, 0.12, and 0.15 mg of digitonin/mg of protein. Succinate oxidase activity was measured in 42 mM mannitol-8 mM Hepes, pH 7.2, with 5.7 mM succinate, 2.3 μM cytochrome *c*, and 10 μM carbonyl cyanide *m*-chlorophenylhydrazone. Cytochrome oxidase activity was monitored by the subsequent addition of ascorbate and TMPD to final concentrations of 2.8 and 0.56 mM, respectively.

centers, shortening the path that ubiquinone and cytochrome *c* would have to travel to accomplish diffusion-mediated electron transfer. However, our findings with digitonin suggest that ubiquinone mobility may be reduced concomitant with patching of protein. This raises some question as to the mode of action of cholesterol as well.

Cytochrome c Diffusion. A number of lysine residues surrounding the heme crevice are established to be important in the binding of cytochrome *c* to cytochrome oxidase and cytochrome bc_1. Since the isothiocyanate-conjugated dyes used to prepare fluorescent cytochrome *c* react with lysines, careful purification and characterization of the cytochrome *c* derivatives are essential to ensure that the mobilities obtained are representative of those of the native protein.

The modification of lysine residues surrounding the heme crevice was minimized by reacting a slight excess of tetramethylrhodamine isothiocyanate or morpholinorhodamine isothiocyanate with cytochrome *c* bound to CM-cellulose. Following the removal of free dye from cytochrome *c* by gel filtration, singly-modified derivatives were separated from native and multilabeled cytochrome *c* by chromatography on CM-cellulose. Rechromatography of the singly-modified derivatives obtained from reaction with either dye gave similar elution profiles, as shown for the morpholinorhodamine derivatives in Figure 3. Cytochrome *c* from peak D was found to be highly fluorescent (excitation maximum = 510 nm; emission maximum = 550 nm) and exhibited nativelike biphasic kinetic behavior with two apparent Michaelis constants ($K_{m_1} = 8.3 \times 10^{-9}$ M; $K_{m_2} = 2.1 \times 10^{-7}$ M) when assayed with cytochrome oxidase in rat liver mitochondria (Figure 3). Two-dimensional chymotryptic peptide maps revealed that peak D contained a mixture of derivatives modified at lysines-22, -39, and -99 (data not shown). A peak containing a similar mixture of derivatives was obtained by reaction with tetramethylrhodamine isothiocyanate. When either of these derivative mixtures was stored for long periods of time (9 months) at −20 °C, some loss of activity and fluorescence was

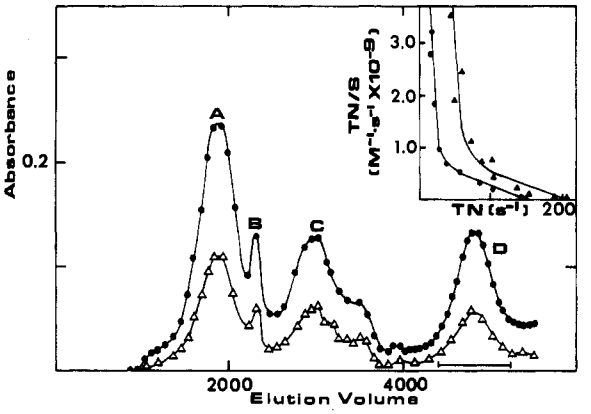

FIGURE 3: Purification and kinetic characterization of morpholinorhodamine (MR) cytochrome *c*. Elution profile of MR cytochrome *c* mixture chromatographed on CM-cellulose as described under Materials and methods. Elution volume is in milliliters. Absorbance was monitored at 410 (●) and 280 nm (△). Inset: Kinetics of native cytochrome *c* (▲) (0.0127–6.45 μM) and cytochrome *c* derivative peak D (●) (0.0055–5.03 μM) with rat liver mitoplasts (3.13×10^{-2} nmol of cyt aa_3) in 1.8 mL of 250 mM sucrose and 25 mM Tris–cacodylate, pH 7.9. Turnover numbers (TN) are activities expressed as electrons per second per cytochrome aa_3. *S* is the molar concentration of cytochrome *c*.

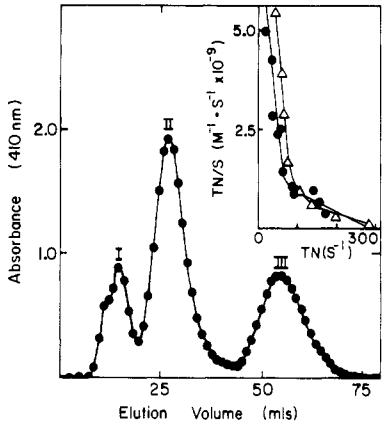

FIGURE 4: Purification and kinetic characterization of tetramethylrhodamine (TMR) cytochrome *c* modified at Lys-39. Rechromatography of peak D from a TMR cytochrome *c* preparation on CM-cellulose after prolonged storage. Inset: Kinetics of native cytochrome *c* (0.008–2.63 μM) (△) and cytochrome *c* derivative peak III (0.0044–0.482 μM) (●) with rat liver mitoplasts (2.5×10^{-2} nmol of cyt aa_3). Assay conditions were as described in Figure 3.

observed. The elution profile from rechromatography of tetramethylrhodamine cytochrome *c*, peak D (after storage), is shown in Figure 4. Peaks I and II showed diminished electron-transfer activity, but peak III had good fluorescence and native activity with cytochrome *c* depleted mitochondria ($K_{m_1} = 9.4 \times 10^{-9}$ M; $K_{m_2} = 2.0 \times 10^{-7}$ M) (Figure 4). A chymotryptic peptide map of peak III (Figure 5) shows that the primary site of modification is at lysine-39. Derivatives of cytochrome *c* at this position on the molecule have minimal effects on the activity and binding of cytochrome *c* with cytochrome oxidase (Ferguson-Miller et al., 1978).

Giant mitoplasts prepared in the absence of digitonin were labeled with fluorescent cytochrome *c* (either the Lys-22/Lys-99/Lys-39 mixture of morpholinorhodamine derivatives or the tetramethylrhodamine derivative at position 39) under low ionic strength conditions where cytochrome *c* was bound in about 10-fold excess of the normal 1:1 or 2:1 ratio. Thus, the majority of the cytochrome *c* was interacting with nonspecific sites on the mitochondrial membrane, and the diffusion rates observed reflect those expected for cytochrome *c* diffusing

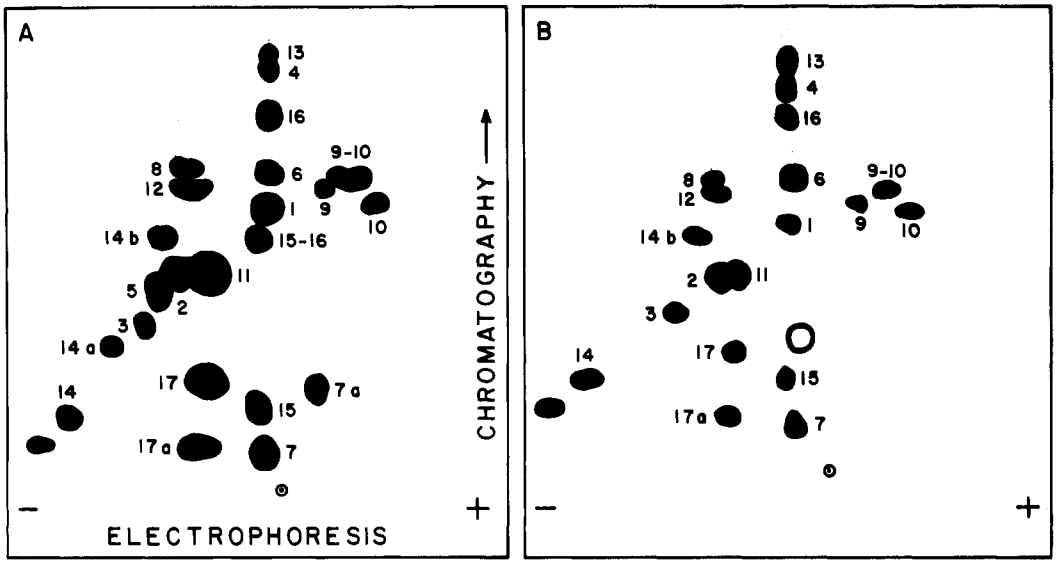

FIGURE 5: Chymotryptic peptide map of native cytochrome *c* (A) and tetramethylrhodamine cytochrome *c* modified of Lys-39 (B). The non-heme-containing peptides were stained with ninhydrin and are assigned according to Brautigan et al. (1978b). The TMR-modified peptide is indicated by the encircled spot; 0.2–0.5 mg of digested cytochrome *c* was applied for each map. Arginine-containing peptides were also identified for the native cytochrome *c* by using a modification of the Sakaguchi procedure reported by Easley (1965). The complete disappearance of peptide 5 and the appearance of a new peptide with a more negative charge are indicative of a modification at position 39.

Table I: Diffusion Coefficients for Mitochondrial Membrane Components

component	probe	buffer	diffusion coefficient[a] (cm^2/s)	% recovery
lipid	NBD-PE	42 mM mannitol and 8 mM Hepes, pH 7.2	$6.0\ (\pm 2.1) \times 10^{-9}$	>90
cyt aa_3	MR rabbit anti-cyt aa_3 IgG	42 mM mannitol and 8 mM Hepes, pH 7.2	$1.5\ (\pm 1.0) \times 10^{-10}$	85
cyt *c*	TMR cyt *c* modified at Lys-39	42 mM mannitol and 8 mM Hepes, pH 7.2	$3.8\ (\pm 1.6) \times 10^{-10}$	89
	MR cyt *c* (Lys-22, -39, and -99 mixture)	42 mM mannitol and 8 mM Hepes, pH 7.2	$3.5\ (\pm 1.5) \times 10^{-10}$	92
		25 mM Tris–cacodylate	$7.4\ (\pm 4.1) \times 10^{-10}$	84

[a] Values are expressed as ± standard deviation. Abbreviations: MR, morpholinorhodamine; TMR, tetramethylrhodamine.

between redox centers. FRAP measurements on these mitochondria in 42 mM mannitol–8 mM Hepes, pH 7.2, showed complete monophasic recovery for both cytochrome *c* derivatives with a diffusion coefficient of 3.5×10^{-10} cm^2/s for the mixture and 3.8×10^{-10} cm^2/s for the derivative of cytochrome *c* at position 39 (Figure 6). This value confirms our previous results with a less highly purified derivative (Hochman et al., 1982, 1983a). At higher ionic strength (25 mM Tris–cacodylate, pH 7.9), the observed diffusion coefficient was 7×10^{-10} cm^2/s (see also Table I).

Cytochrome Oxidase Diffusion. The mobility of cytochrome oxidase was investigated by using fluorescently labeled antibodies prepared and purified as described under Materials and Methods. To avoid cross-linking of the oxidase in the membrane, mitochondria were incubated with a large excess of antibodies and washed 3 times to remove any nonspecifically bound forms. Diffusion measurements on these mitochondria showed complete monophasic recovery, indicating that there was no significant cross-linking induced by the antibodies. From these data, we calculated a diffusion coefficient of 1.5×10^{-10} cm^2/s for cytochrome oxidase. Monovalent Fab fragments prepared from these antibodies gave the same average diffusion coefficient, but since their fluorescence was much lower, the error in the data was greater.

The results of the FRAP measurements on giant mitoplasts prepared by sonication are summarized in Table I. One interesting feature of these results is that in spite of the high protein content of the mitochondrial inner membrane the diffusion coefficients obtained for cytochrome oxidase and for phosphatidylethanolamine in these osmotically swollen membranes approximate the values for lipids and integral proteins

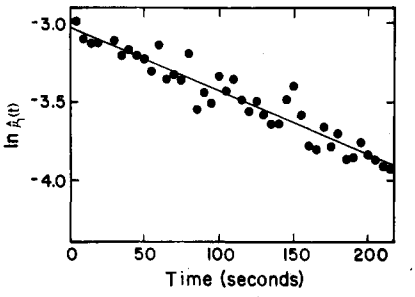

FIGURE 6: Recovery curve for tetramethylrhodamine cytochrome *c* modified at Lys-39 on a swollen giant mitoplast in 42 mM mannitol and 8 mM Hepes adjusted with Tris to pH 7.2. Scans were taken every 5 s. $D = 3.8 \times 10^{-10}$ cm^2/s for this experiment.

in membranes containing a lower protein concentration [see McCloskey & Poo (1984)].

Does Electron Transfer Occur by a Random Diffusion Mechanism? In a previous report, we made a preliminary calculation of the minimal diffusion coefficient required for cytochrome *c* to accomplish electron transfer by a random diffusion mechanism at rates observed under the conditions of our mobility measurements. Equating the total path length for a single turnover event to the root mean square displacement of cytochrome *c*, we calculated a coefficient of 4×10^{-9} cm^2/s. This calculated did not take into account the size of the reacting center or the mobility of the redox partners of cytochrome *c*. Since we have obtained a diffusion coefficient for cytochrome oxidase measured under the same conditions as cytochrome *c*, we can now take these factors into account. The concentration of cytochrome oxidase in the inner mem-

branes of rat liver mitochondria was accurately determined by taking osmotically shocked mitoplasts (diluted into 10 mM Tris–phosphate at <5 mg of protein/mL), sonicating extensively, and pelleting the membrane fragments at 40000*g* for 1 h. The membranes had 0.4–0.45 nmol of heme *a*/mg of protein. A similar value is obtained by freeze–thawing mitoplasts, washing, and pelleting the membranes. If a low concentration of detergent (e.g., 5 mM lauryl maltoside) is used to disrupt the mitoplasts, significant loss of intrinsic membrane proteins (particularly cytochrome bc^1) is observed, with retention of all the oxidase, leading to a heme *a* to protein ratio of 0.7 (Thompson, 1984). Using the former value for the concentration of heme *a* and the approach outlined previously (Hochman et al., 1982), we calculated that oxidase comprises 4.5% of the total protein in the rat liver inner membrane and is present at a concentration of 3.8×10^{10} monomers/cm^2. Since only 10–15% of the cytochrome *c* is reduced under normal steady-state conditions in mitochondria (Nicholls, 1974, 1976), the concentration of cytochrome *c* involved in transferring electrons to oxidase is ($3.8–5.8 \times 10^9$ molecules/cm^2. If we assume that the target area for cytochrome *c* reacting with cytochrome oxidase has a radius of 40 Å (4×10^{-7} cm), then using the equation of Hardt (1979):

$$\Phi = 2\pi C_{aa_3} C_c \left\{ \frac{D_{aa_3}}{\ln [(\pi C_c)^{-1/2}/a]} + \frac{D_c}{\ln [(\pi C_{aa_3})^{-1/2}/a]} \right\}$$

where Φ = the frequency of collisions between cytochrome *c* and cytochrome oxidase and *a* = the radius of the collisional domain within which cytochrome *c* can transfer an electron to cytochrome oxidase. We calculate that for the steady-state electron-transfer rate observed under the conditions of the diffusion measurements [turnover number (TN) = 20 electrons (cytochrome $aa_3)^{-1}$ s^{-1}; $\Phi = 7.6 \times 10^{11}$ electrons cm^{-2} s^{-1}], the *minimal diffusion coefficient* that would be compatible with a random diffusion measurement is

$$D_c = (1.0–1.6) \times 10^{-9} \text{ cm}^2/\text{s}$$

To make this calculation, we assume that the electron-transfer reactions occur by random collisions and do not involve the formation of any long-lived complexes (i.e., dissociation rates of cytochrome *c* from the complexes are very fast) and that every collision between a reduced cytochrome *c* and cytochrome oxidase will result in an electron-transfer event (100% efficiency). These assumptions bias the calculation in the direction of a considerable underestimate of the required diffusion coefficient, giving the slowest possible mobility for cytochrome *c* that would be compatible with observed rates of electron transfer.

While the calculated diffusion coefficient is a minimal estimate, the experimental determined coefficient is biased in the direction of a maximal value, since potential steric hindrances are removed by swelling the mitochondria and excess cytochrome *c* is used for the measurements. Normal unswollen mitochondria have cristae invaginations in which the inner membranes are in close apposition, separated by approximately 150–200 Å (Srere, 1982). Intrinsic proteins extend into this space as much as 70 Å, and other proteins appear to be densely packed on the outer and matrix sides of the membrane (Sjostrand, 1979; Williams, 1983). Removal of these impediments to short- and long-range movements should give rise to upper limit estimates of mobilities. The use of excess cytochrome *c* to obtain accurate fluorescent measurements makes it impossible to distinguish the difusional characteristics of the small amount of specifically bound cytochrome *c*. Thus, the measured diffusion coefficients do not take into account the potentially limited rates of dissociation of cytochrome *c* from its redox partners. This is particularly important when considered the influence of ionic strength on diffusion rates, since binding to the lower affinity, nonspecific sites on the membrane is likely to be more strongly affected by ionic strength than binding to the high-affinity sites on the redox partners (Vanderkooi et al., 1973; Ferguson-Miller et al., 1979). Indeed, a preliminary report indicates that when cytochrome *c* is used in amounts stoichiometric with cytochrome oxidase and the mitochondria are intact and unswollen, cytochrome *c* diffusion is greatly diminished and is insensitive to ionic strength (Maniara et al., 1984). Thus, even though our calculation is biased in the direction of a lower limit estimate and our measurements are made under conditions favoring an upper limit value, a comparison of these diffusion coefficients indicates that the mobility of cytochrome *c* is not sufficient to explain electron transfer by a completely random diffusion mechanism. An electron-transfer rate of only 5 to 8 electrons per second (compared to an observed rate of 20 electrons per second) could be achieved by a collisional mechanism with the measured diffusion coefficients (Table I).

We have previously proposed a model (Hochman et al., 1982, 1983a) which is consistent with the present findings and with the results of other investigators. In this model, electron transfer can occur by random diffusion, but more rapid rates are achieved by formation of transitory functional aggregates (dynamic aggregates) among the electron-transfer chain components. In the case of cytochrome *c*, physical association between cytochromes bc_1 and aa_3 could create a limited domain in which cytochrome *c* movement is restricted, thereby increasing the efficiency of electron conductance. In *Paracoccus denitrificans*, the components of the respiratory chain including cytochrome *c* appear to be tightly associated, suggesting that even though high rates of electron transfer occur in the system (Erecinska et al., 1979), the diffusion of cytochrome c may be severely restricted. The observations of Cherry and co-workers (Kawato et al., 1980, 1982) indicate that the respiratory complexes of mammalian mitochondria are to a large extent immobilized on a short time scale, suggesting a high degree of aggregation in the native membrane. The authors ascribe this phenomenon to nonspecific aggregation, but it seems more reasonable from a physiological standpoint that functional aggregates would predominate.

Although the efficiency of electron transfer may be increased by formation of specific associations among the complexes, it is clear that the structural features of cytochrome *c* are also compatible with two- or three-dimensional diffusion, and this mode of activity may be physiologically important as well. Indeed, Froud & Ragan (1984) recently reported that cytochrome *c* restores ubiquinol oxidase activity in a manner consistent with a mobile pool mechanism in a reconsitituted system containing complexes III and IV and excess cytochrome *c*. While this study demonstrates the feasibility of such a mechanism, it does not show that this is the exclusive or even predominant mode of electron transfer by cytochrome *c* in native mitochondria, where stoichiometric amounts of cytochrome *c* are sufficient to restore the full activity of the respiratory chain (Nicholls, 1974, 1976).

In contrast to cytochrome *c*, a 10-fold excess of ubiquinone over its redox partners is found in the mitochondrial membrane, making a pool function for this mediator more likely. Applying the analysis used to determine the minimal diffusion coefficient for cytochrome *c*, we find that for 1.9×10^{10} monomers of cytochrome bc_1/cm^2 and 10% of the ubiquinone reduced (Kroger & Klingenberg, 1975) during steady-state

electron transfer, the minimum diffusion coefficient for ubiquinone is 6.4×10^{-10} cm^2/s (1.9×10^{-9} cm^2/s if cytochrome bc_1 is present as a dimer). If we assume that ubiquinone diffusion rates are similar to that of lipid[2] (6×10^{-9} cm^2/s), then the diffusion coefficient is compatible with a random collision mechanism. However, observations indicative of direct interactions of cytochrome bc_1 with the NADH dehydrogenase complex (Ragan & Heron, 1978) and succinate dehydrogenase (Yu et al., 1974) also justify consideration of the dynamic aggregate model for this part of the respiratory chain.

To evaluate how the proposed model may apply to the physiological functioning of mitochondria, it is important to consider the effects of high protein concentrations in the matrix and intermembrane spaces on the mobilities of membrane-bound components. It has been proposed that the matrix of mitochondria has a very high protein concentration when in the condensed configuration (Srere, 1982). This protein crowding could hinder lateral and rotational mobility of the integral complexes, resulting in reorganization on a time scale much larger than that of an electron-transfer event. Association and dissociation of the complexes in this time frame would be compatible with a regulatory function mediated by factors that affect the lifetime of the aggregated state. Possible modulators might include hormone-induced phosphorylation, changes in calcium levels, or alterations in the membrane potential itself. An analogy is provided by the studies of Kaback and co-workers (Goldkorn et al., 1984) which indicate that the state of aggregation of a bacterial membrane protein is modulated by the membrane potential. In mitochondria, a high-membrane potential might favor a disaggregated state, while at a low-potential aggregated complexes would predominate, giving rise to more efficient electron and energy transfer.

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[2] Preliminary evidence using fluorescently labeled ubiquinone 10 indicates that its diffusion is also slower in digitonin-treated mitochondrial membranes compared to inner membrane prepared in the absence of digitonin.

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