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Protein-Protein Interactions of Cytochrome Oxidase in Inner Mitochondrial Membranes

THE EFFECT OF LIPOSOME FUSION ON PROTEIN ROTATIONAL MOBILITY*

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Rotational diffusion of cytochrome oxidase in the inner membrane of rat liver mitochondria was measured by detecting the decay of absorption anisotropy after photolysis of the heme a_3•CO complex by a vertically polarized laser flash. As in previous experiments with beef heart mitochondria (Kawato, S., Sigel, E., Carafoli, E., and Cherry, R. J. (1980) *J. Biol. Chem.* 255, 5508-5510), co-existence of rotating cytochrome oxidase (mean rotational relaxation time, ϕ, of 700 to 1400 μs) and immobilized cytochrome oxidase (ϕ > 20 ms) was observed in mitochondria and mitoplasts.

The effect of lipid/protein ratio by weight (L/P) on the relative proportions of mobile and immobile cytochrome oxidase was investigated following the fusion of soybean phospholipid vesicles with mitoplasts. The fusion procedure yielded four separate fractions upon sucrose density gradient centrifugation with L/P as follows: 0.3 in Pellet, 0.7 in Band 3, 1.5 in Band 2, and 3.0 in Band 1. The percentage of rotationally mobile cytochrome oxidase (ϕ = 700 to 1000 μs) in each of the different bands was found to be 16% in Pellet, 25% in Band 3, 47% in Band 2, and 76% in Band 1 at 37 °C. The dependence of the amount of mobile cytochrome oxidase on L/P indicates that the fraction of aggregated protein progressively decreases with decreasing concentration of proteins in the membrane. Thus, the large immobile fraction of cytochrome oxidase in mitochondrial inner membranes can be explained by nonspecific protein aggregation which is a consequence of the low L/P. The decrease in the mobile fraction in Pellet compared with mitoplasts was shown to be due to the pH 6.5 incubation used for fusion.

Lateral and rotational mobilities of component membrane proteins may play a significant role in oxidative phosphorylation in the inner membrane of mitochondria (1). It is important to establish whether catalytically interacting individual redox proteins are distributed randomly or in association as several structurally distinct complexes, or as complete respiratory chains (2). Hackenbrock and co-workers investigated lateral collisions between component proteins in the respiratory chain by measuring the kinetics of electron transfer in phospholipid-enriched inner membranes of rat liver mitochondria (3, 4). They observed decreased rates of electron transfer for a variety of steps in the respiratory chain according to the increase in average distance between integral membrane proteins. We have shown independent rotational and lateral diffusion of cytochrome bc_1 complex and cytochrome oxidase in phospholipid vesicles (5).

Rotational mobility of cytochrome oxidase has previously been examined extensively in both the inner mitochondrial membrane (6, 7) and reconstituted phospholipid vesicles (5, 8, 9). Protein rotation measurements are particularly sensitive to protein-protein interactions (10, 11). They can be used to quantify mobile and immobile fractions of cytochrome oxidase on the basis of a "rotation-about-membrane normal" model (12). In beef heart mitochondrial membranes, co-existence of mobile and immobile cytochrome oxidase was observed. The immobile population was about 60% of the total oxidase. In phospholipid vesicles, the amount of immobile cytochrome oxidase was negligible at L/P[1] = 30 but increased to about 20% at L/P = 5. These results suggest that the large immobile fraction in mitochondria may consist of protein aggregates which are a consequence of the low L/P (≃0.4) of the inner membrane (5, 6).

To test this hypothesis, we have used the procedure of Schneider *et al.* (3, 4) for enriching phospholipids in the inner membrane of mitochondria. This liposome-inner membrane fusion method has enabled us to measure the rotational mobility of cytochrome oxidase as a function of L/P in the mitochondrial inner membrane itself. Here we present the results and implications of these observations with particular emphasis on the effect of L/P on protein aggregation.

EXPERIMENTAL PROCEDURES

Preparations of Mitochondria and Mitoplasts—Liver mitochondria were isolated from male Wistar rats according to Pederson *et al.* (13) and suspended in Buffer A consisting of 220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.5 mg of BSA/ml and KOH (pH 7.4). Mitoplasts were prepared by incubation with digitonin essentially as described by Hackenbrock (14), and washed and suspended in a 7.5 times diluted BSA-free buffer A (buffer B).

Fusion of Mitoplasts with Liposomes—Liposomes were prepared from 1.5 g of soybean phospholipids in 7.5 ml of buffer B by sonication with a Branson-type sonifier with pulsed mode for 1 h at 4 °C. Mitoplasts were fused with liposomes following the procedure of Schneider *et al.* (3, 4). 1.25-ml aliquots of liposomes were added at 10-min intervals to 7.5 ml of mitoplast suspensions (100 to 150 mg in protein) with constant stirring at 30 °C. After each addition, the pH was adjusted immediately to pH 6.5 with HCl. After 6 additions (total time of 60 min), the pH was adjusted to 7.4 with KOH and the mitoplast-liposome suspensions were cooled on ice.

Aliquots of 5 ml of the mixed mitoplast-liposome suspension per tube (40 ml) were centrifuged in a discontinuous sucrose density gradient (0.6, 0.75, 1.0, and 1.25 M sucrose in buffer B) at 70,000 × *g*

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L/P, lipid/protein (w/w) ratio; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

for 15 h at 2 °C. Liposomes remained on the top of the gradient, while fused membranes were separated into four distinct fractions designated Band 1, Band 2, Band 3, and Pellet, from least to most dense. All four bands were washed with buffer B to remove excess sucrose.

Assay of Enzymes—NADH oxidase, succinate oxidase, and duroquinol oxidase activities were determined polarographically by measuring the oxygen consumption with a Clark oxygen electrode for 0.25 mg of protein in 0.8 ml volume at 25 °C. The incubation medium contained 120 mM KCl, 2 mM Hepes, 10 mM K-phosphate (pH 7.4), 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 5 μM cytochrome *c*. The oxygen consumption activity was measured in the presence of the following substrates and inhibitors. (*a*) NADH oxidase: 2 mM NADH, 3 μM rotenone, (*b*) succinate oxidase: 5 mM Na-succinate, 2 μM antimycin, (*c*) duroquinol oxidase: 0.6 mM freshly reduced duroquinol, 2 μM antimycin, (*d*) cytochrome *c* oxidase: 1.25 mM Na-ascorbate, 62 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, 1 mM KCN. All oxidase assays were initiated by the addition of membrane suspensions except for duroquinol oxidase assay which was initiated by duroquinol. Antimycin and KCN were used to correct for the autooxidation of duroquinol.

Determination of Cytochromes—Dithionate-reduced minus air-oxidized difference spectra were obtained with an Aminco DW-2a spectrophotometer. Concentrations of cytochrome hemes *b*, c_1, *c*, *a* were calculated by simultaneous equations according to the method of Williams (15), employing the extinction coefficients from Schneider *et al.* (4). Cytochrome *a* can be independently determined with $\Delta A_{605-630}$ after dithionate reduction using an extinction coefficient of 13.1 mM^{-1} cm^{-1} (16).

Gel Electrophoresis—A slightly modified method of Laemmli (17) was used. Membranes were incubated for 3 min at 95 °C in 10 mM Na-phosphate buffer (pH 7.0) containing 10% glycerol (v/v), 2.5% SDS (w/v), 3.25 mM dithiothreitol and 0.5% bromphenol blue (w/v). The sample was applied on a step (12% and 15%) polyacrylamide slab gel (1.5 mm thick). Acrylamide including 0.5% polyacrylamide was polymerized in 325 mM Tris-HCl buffer (pH 8.8) containing 2 mM EDTA, 0.1% SDS. The electrode buffer consisted of 50 mM Tris, 380 mM glycine, 0.1% SDS, and 1.8 mM EDTA. The sample was fixed with 10% (w/v) trichloroacetic acid, stained with 0.25% Coomassie blue and destained with 7.5% acetic acid in aqueous methanol. BSA, ovalbumin, chymotrypsinogen, cytochrome *c*, and cytochrome oxidase were used for molecular weight standards. Rat liver cytochrome oxidase was purified according to the method of Ades and Cascarano (18).

Electron Microscopy—Samples were jet-frozen with liquid propane using a Balzer's Cryo-jet (19). Freeze fracturing was carried out in a Balzer's BAF 300 freeze-etching apparatus. Specimens were replicated with Pt/C and were examined in a Philips EM 301 electron microscope.

Determination of Protein and Phosphorus—Protein concentration was determined with the method of Lowry *et al.* (20) in the presence of 1% SDS and with the biuret method (21). In both cases, BSA was used as standard. Lipid phosphorus was measured according to the method of Chen (22) after acid hydrolysis with perchloric and sulfuric acid.

Materials—Asolectin was purchased from Associated Concentrates, Inc. Horse heart cytochrome *c* (grade VI) and BSA were obtained from Sigma. Duroquinol and digitonin were purchased from Fluka. Other chemicals used were obtained in the highest purity available commercially.

Rotational Diffusion Measurements—For rotational diffusion measurements, 0.77 g of sucrose was dissolved in 515 μl of membrane suspensions resulted in 60% (w/w) sucrose solution. The final protein concentration was 2 to 5 mg. Samples were reduced by 25 mM ascorbate and 50 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, and slowly bubbled for 1 min with CO.

The flash photolysis apparatus used for rotation measurements is described in detail elsewhere (23). Briefly, the sample was excited at 590 nm by a vertically polarized flash of duration 1 to 2 μs from a dye laser (Rhodamine 6G in methanol). Absorbance changes due to photolysis of the heme a_3·CO complex were measured at 446 nm. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, and the total absorbance change, $A(t)$, given by

$$r(t) = [A_V(t) - A_H(t)]/[A_V(t) + 2A_H(t)] \quad (1)$$

$$A(t) = A_V(t) + 2A_H(t) \quad (2)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorbance changes for vertical and horizontal polarization at time t after the flash.

In each experiment, about 1000 signals were averaged using a Datalab DL 102A signal averager and Hewlett-Packard HP 9825A desk top computer. Samples were measured in 60% sucrose solution in order to reduce light scattering. Since mitochondria, mitoplasts, and lipid-enriched inner membranes of mitochondria are larger than 0.5 μm in diameter, these membranes are completely immobilized within the measurement time range of 2 ms (*i.e.* vesicle tumbling slower than 100 ms).

Analysis of Absorption Anisotropy—Detailed theoretical and experimental considerations of rotational diffusion and protein-protein interactions of cytochrome oxidase are described elsewhere in detail (5, 12). Briefly, decays in absorption anisotropy, $r(t)$, were analyzed based on a model in which rotation of heme proteins occurs only about the membrane normal. Evidence that this model is applicable to cytochrome oxidase has been presented previously (5).

$$r(t)/r(0) = 3\sin^2\theta_N\cos^2\theta_N\exp(-t/\phi_\parallel) + \tfrac{3}{4}\sin^4\theta_N\exp(-4t/\phi_\parallel) + \tfrac{1}{4}(3\cos^2\theta_N - 1)^2 \quad (3)$$

where $\phi_\parallel$ is the rotational relaxation time and θ_N is the tilt angle of the heme plane from the membrane plane. Equation 3 expresses rotation of a single rotating species with 4-fold symmetry of the heme plane. However, multiple populations of cytochrome oxidase have been shown to occur in mitochondrial membranes (5, 6). Although such multiple populations are difficult to resolve, their existence can be inferred by fitting the data by the following general equation

$$r(t) = r_1\exp(-t/\phi) + r_2\exp(-4t/\phi) + r_3 \quad (4)$$

and comparing r_1, r_2, and r_3 with the theoretical values of Equation 3. In the case of co-existence of mobile and immobile populations, the fraction of mobile cytochrome oxidase, f_m, can be estimated by

$$r_3/r(0) = 0.25f_m + (1 - f_m) \quad (5)$$

where we assume that the theoretical time-independent term in Equation 3 is 0.25 ($\theta_N = 90°$). This is justified by the finding that the heme a_3 plane is approximately perpendicular to the membrane plane (5, 24).

Curve fitting of the data by Equation 4 was accomplished by a HP 9825 computer. It should be noted that in Equation 3 and 4, $r(t)/r(0)$ does not depend on the intensity of the photoselecting flash and only $r(0)$ depends on the flash intensity (12). Therefore, $r(t)$ values obtained at slightly different excitation intensities may be normalized to the same $r(0)$ for direct comparison in figures.

RESULTS

Structural and Chemical Characterization of Phospholipid-enriched Inner Membranes of Mitochondria

Four separate inner membrane fractions were obtained with gradient centrifugation in sucrose after liposome-inner mitochondrial membrane fusion as described under "Experimental Procedures." The above four different inner membrane fractions were designated Bands 1, 2, 3, and Pellet as shown in Table I. L/P calculated from the measured lipid phosphorus and protein contents showed a progressive increase from Pellet (L/P ~ 0.3) to Band 1 (L/P ~ 3.0).

A dilution of membrane proteins with the present fusion procedures was demonstrated by freeze-fracture electron microscopy. Fig. 1 shows typical examples of lipid-enriched inner mitochondrial membranes jet-frozen from room temperature (cooling rate ~10^4 °C/s). A progressive increase in the average distance between intramembrane particles (integral proteins) is observed from Pellet to Band 1. In Band 1 almost no large protein aggregates are visible.

The distribution of cytochromes over Bands 1 to 3 and Pellet is summarized in Table I. The heme a content was distributed heterogeneously and enriched more in Bands 2 and 3 than other membrane fractions. A similar tendency was observed for cytochromes *b* and c_1. The content of the above three cytochromes in fused inner membranes was larger than that in mitoplasts, probably because matrix proteins were lost during the fusion, centrifugation, and washing procedures. In

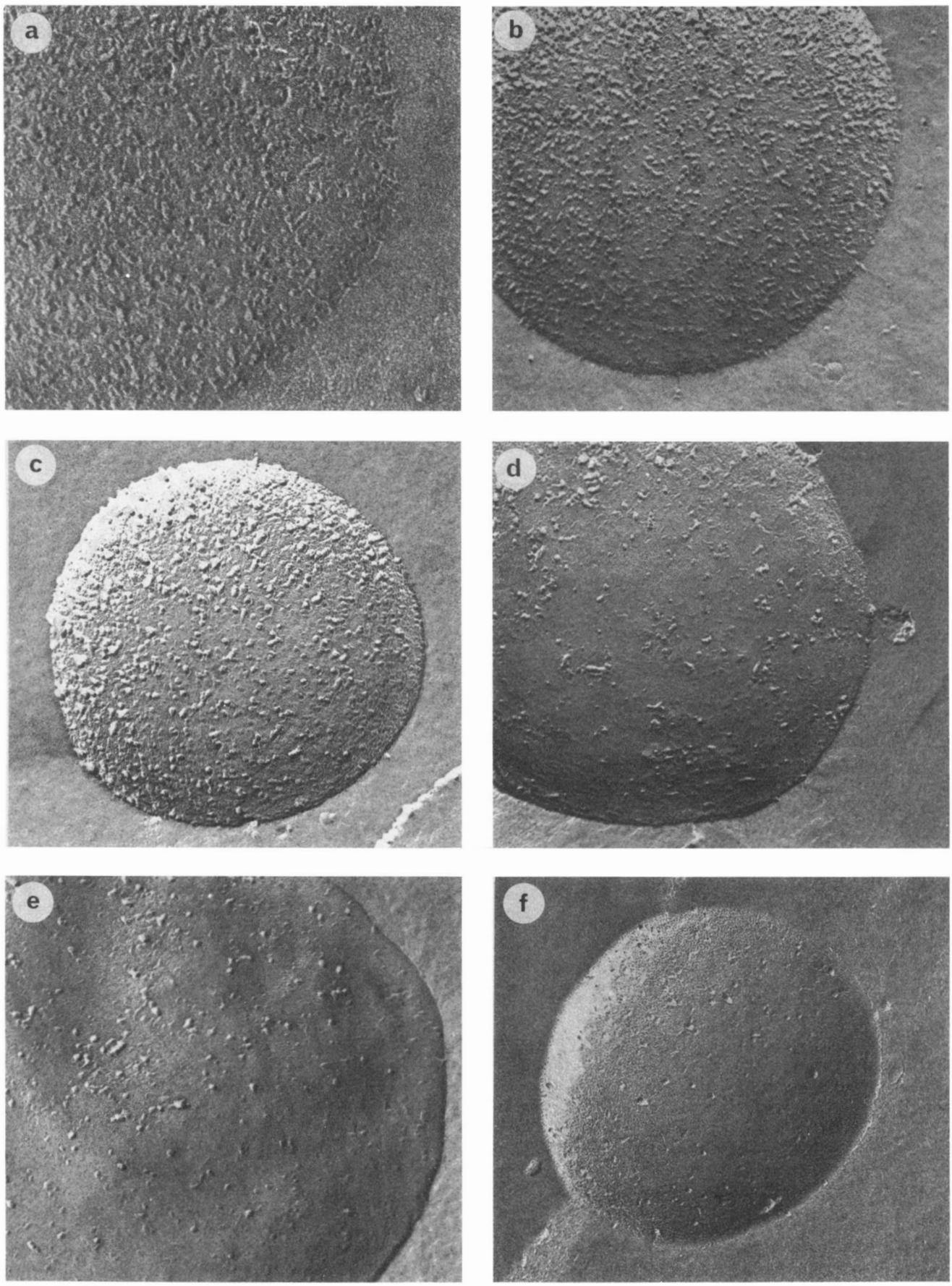

FIG. 1. **Freeze-fracture electron micrographs of four different mitochondrial inner membrane fractions obtained by fusion with liposomes followed by density gradient centrifugation in sucrose.** *a*, mitoplasts (control); *b*, Pellet; *c*, Band 3; *d*, Band 2; *e* and *f*, Band 1. *f* is a concave face and others are all convex faces. Samples were jet-frozen from room temperature. Average distance between intramembrane particles (integral proteins) increases progressively from Pellet to Band 1. × 112,000.

TABLE I

Compositional analysis of mitochondrial inner membranes after fusion with liposomes

The data are averaged from several independent experiments.

Membrane	Sucrose density (w/v)	Protein yield[a]	μmol lipid phosphorus/ mg protein	L/P[b] (w/w)	nmol heme a/mg protein	*b*/a[c]	c_1/a[c]	*c*/a[c]
	%	%						
Mitoplasts			0.28	0.2	0.21	0.55	0.34	0.50
Pellet		64	0.40	0.3	0.27	0.54	0.26	0.12
Band 3	34	6.3	0.93	0.7	0.68	0.50	0.29	0.10
Band 2	28	4.2	1.89	1.5	0.75	0.60	0.42	0.10
Band 1	21	2.0	3.80	3.0	0.53	0.48	0.34	0.11

[a] 23.5% of proteins were recovered in the liposome layer on the top of the sucrose density gradient after centrifugation.

[b] Lipid/protein weight ratio calculated from lipid phosphorus/protein ratio assuming that the average weight of phospholipid is 800/phosphorus.

[c] Molar ratios of cytochromes *b*, c_1, and *c* to cytochrome *a*.

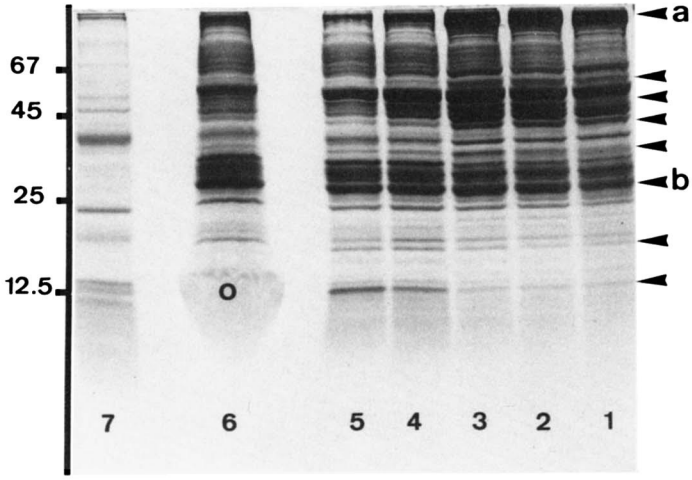

FIG. 2. **SDS-polyacrylamide gel electrophoresis of mitochondria, mitoplasts and four different inner membrane fractions obtained by the liposome fusion procedures.** *1*, mitochondria; *2*, mitoplasts; *3*, Pellet; *4*, Band 3; *5*, Band 2; *6*, Band 1; *7*, purified rat liver cytochrome oxidase. *Arrows* indicate protein bands which differ in density from one membrane fraction to another. o in Band 1 is due to contamination by soybean phospholipids. Numbers along the vertical axis are the molecular weight ($\times 10^{-3}$).

contrast, cytochrome *c*, a loosely bound membrane protein, was lost by about 80% after the fusion procedures.

The distribution of all membrane proteins in lipid-enriched inner mitochondrial membranes was examined by SDS-polyacrylamide gel electrophoresis. Fig. 2 shows a typical example where each membrane fraction contains the same amount of protein in weight. Relative amounts of several Coomassie blue-stained protein bands (indicated with *arrows* in Fig. 2) differ from one membrane fraction to another. A heavy molecular weight protein band (*arrow a*) progressively becomes weaker from mitoplasts to Band 1 fraction. All subunits of cytochrome oxidase are concentrated in Bands 2 and 3 while a decreased amount of cytochrome oxidase is observed in other membrane fractions. In contrast, no significant difference in the relative content is observed for the band of $M_r \sim$ 26,000 (*arrow b*) which probably corresponds to the ADP/ATP translocator (25, 26). The above results were reproducible for four independent experiments.

Electron Transfer Activities of Complexes I, II, III, and IV

Oxygen consumption activity was used to investigate the effect of lipid-enrichment of inner mitochondrial membranes on electron transfer rates. Since the majority of cytochrome *c* was lost from the inner membrane during the fusion procedures, electron transfer activities were assayed in cytochrome *c* supplemented media as described under "Experimental Procedures." Results are summarized in Table II. NADH oxidase, succinate oxidase, and duroquinol oxidase activities decreased progressively from Pellet to Band 1. Cytochrome oxidase activity decreased from Pellet to Band 3 and remained unchanged over Band 3 to Band 1.

TABLE II

Specific activities of respiratory enzymes in mitochondrial inner membranes after fusion with liposomes

Activities were determined by measuring the oxygen consumption and expressed as turnover numbers in e^-/s/heme a. The data are averaged from several independent experiments.

Membrane	NADH oxidase	Succinate oxidase	Duroquinol oxidase	Cytochrome oxidase
Pellet	54.9	17.6	45	63
Band 3	8.2	10.3	27	31
Band 2	6.3	8.9	15	34
Band 1	3.4	5.4	6	27

Rotational Diffusion of Cytochrome Oxidase

Rotational diffusion of cytochrome oxidase was measured in rat liver mitochondria, mitoplasts, and phospholipid-enriched inner membranes (Fig. 3). Data were analyzed by Equation 4 and decay parameters are summarized in Table III. In all membranes examined, values of $r_3/r(0)$ larger than 0.25 were obtained, indicating co-existence of mobile (ϕ = 700 to 1400 μs) and immobile (ϕ > 20 ms) populations of cytochrome oxidase. Protein-protein interactions were characterized in terms of the percentage of the mobile population of cytochrome oxidase using Equation 5. If the mobile population consists of a single species, $r_1/r_2 = 4\ \mathrm{cot}^2\theta_N$ should be close to zero (since $\theta_N \simeq 90°$ (5, 24)). In all cases examined, however, r_1/r_2 was far from zero, implying that there were multiple rotating species. Therefore, ϕ should be considered as an average value. Any protein microaggregate larger than ~1000 Å in length would contribute to the immobile fraction within the present experimental time range of 2 ms (5).

Effect of Lipid/Protein Ratio in Lipid-enriched Inner Membranes—The large increase in L/P from Pellet to Band 1 progressively increased the fraction of mobile cytochrome oxidase with ϕ = 700 to 1400 μs at both 20 and 37 °C. The mobile population was 0 to 20% in Pellet (L/P ~ 0.3), ~25% in Band 3 (L/P ~ 0.7), ~47% in Band 2 (L/P ~ 1.5) and ~76% in Band 1 (L/P ~ 3) at 37 °C. No significant change was observed in ϕ (700 to 1400 μs) for the rotating population over Pellet to Band 1. In some of the Pellet preparations, no rapidly rotating cytochrome oxidase was detected but only very slow rotation with ϕ = 15 to 20 ms was observed.

Comparison of Mitochondria and Mitoplasts with Pellet—

Co-existence of mobile and immobile populations of cytochrome oxidase was also observed in mitochondria and mitoplasts (Fig. 4). About 26% and 35%, respectively, of cytochrome oxidase were rotating at 37 °C with ϕ = 800 to 1000 μs in mitochondria and mitoplasts. These mobile fractions were much larger than that in Pellet. After the incubation of mitoplasts at pH 6.5, 30 °C for 1 h in the absence of liposomes, a significant decrease in the fraction of mobile cytochrome oxidase ($\phi \sim 1000$ μs) was observed. Sometimes only very slowly rotating cytochrome oxidase (ϕ = 15 to 20 ms) was observed similarly to that in Pellet. Therefore, the increase of the immobile cytochrome oxidase from mitoplasts to Pellet is apparently due to pH 6.5-induced protein aggregation during the fusion procedure.

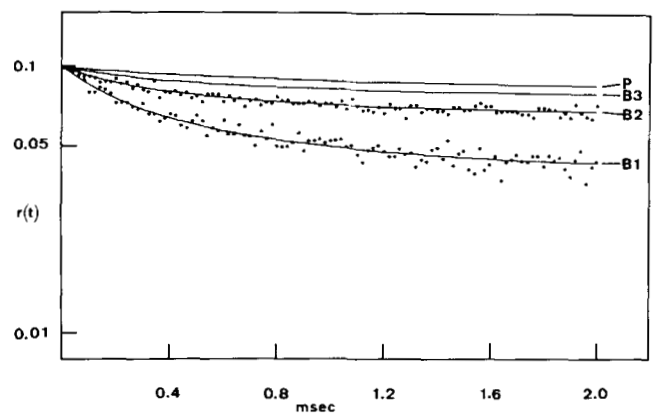

FIG. 3. **Time-dependent absorption anisotropy of cytochrome oxidase in lipid-enriched inner membranes of mitochondria at 37 °C.** Samples (2 to 4 mg in protein) were photolyzed by a vertically polarized laser flash at 590 nm, and $r(t)$ was recorded at 446 nm. All measurements were performed in buffer B containing 60% (w/w) sucrose (25 centipoise at 37 °C). Curves are *P*, Pellet; *B3*, Band 3; *B2*, Band 2; *B1*, Band 1. *Solid lines* were obtained by fitting the data to Equation 4. All curves are normalized to the same $r(0)$ as that of *curve B2* to allow a direct comparison. This is justified because although $r(0)$ depends on the intensity of the photoselecting flash, $r(t)/r(0)$ is independent of the flash intensity (12). Data points of *curves P* and *B3* are omitted for clarity.

Effect of Temperature—Decreasing temperature from 37 to 20 °C decreased the population of the rotating cytochrome oxidase by 5 to 15% in all membranes examined. The above decrease in temperature also increased ϕ (see Table III). The mobilization of cytochrome oxidase by enriching phospholipids in the inner membrane from Pellet to Band 1 was qualitatively similar at 20 °C and 37 °C.

Effect of Freezing of Membranes—Since most of protein rotation measurements were performed with membranes which had been frozen in liquid nitrogen and stored at −80 °C, control experiments were made with freshly prepared membranes. No significant change in $r(t)$ curves was observed within experimental error between frozen-thawed membranes and freshly prepared membranes over mitochondria, mitoplasts, Pellet, and Bands 1 to 3.

Effect of Cytochrome c Addition—The majority of cytochrome *c* was lost from the inner membrane during the fusion

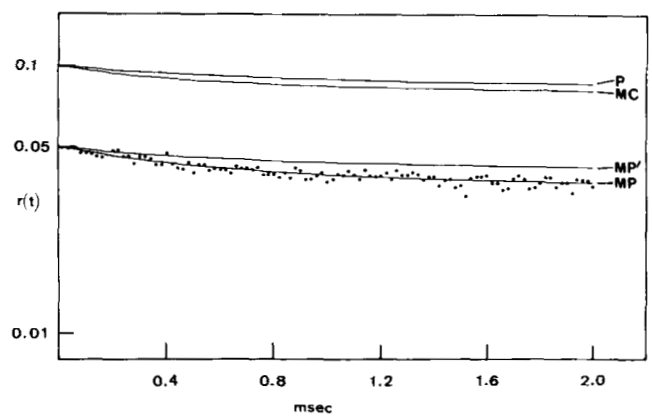

FIG. 4. **Time-dependent absorption anisotropy of cytochrome oxidase in mitochondria, mitoplasts, and Pellet at 37 °C.** Samples contain 4 to 5 mg of protein. Experimental conditions are the same as described in Fig. 3. Curves are *P*, Pellet; *MC*, mitochondria; *MP*, mitoplasts; *MP'*, mitoplasts incubated at pH 6.5 for 1 h then transferred to pH 7.4. *Solid lines* were obtained by fitting the data to Equation 4. Curves are normalized to allow a direct comparison, while *MP* and *MP'* have been displaced vertically for clarity. Data points of curves *P*, *MC*, and *MP'* are omitted for clarity.

TABLE III

Decay parameters of time-dependent absorption anisotropy of cytochrome oxidase in mitochondrial inner membranes analyzed by Equation 4.

Experimental conditions are the same as described in Fig. 3 but with varying temperature.

Membrane	L/P (w/w)	ϕ	r_3/r (0)	Mobile oxidase[a]	r_1/r_2	Temperature
		μs		%		°C
Mitochondria		663 (173)[b]	0.90 (0.04)	13 (5)	~1.4	20
		1073 (29)	0.80 (0.03)	26 (4)	1.6 (0.6)	37
Mitoplasts	0.2	1375 (572)	0.79 (0.03)	29 (5)	18.3 (16.7)	20
		805 (65)	0.74 (0.04)	35 (5)	4.4 (2.8)	37
pH 6.5 mitoplasts[c, d]	0.2	~700	~0.89	~15		20
		~800	~0.85	~20		37
Pellet[d]	0.3	~1000	~0.91	~12		20
		~1000	~0.88	~16		37
Band 3	0.7	1416 (57)	0.86 (0.03)	18 (4)	3.2 (2.3)	20
		806 (171)	0.81 (0.02)	25 (2)	2.8 (0.3)	37
Band 2	1.5	778 (137)	0.69 (0.01)	42 (2)	3.4 (0.6)	20
		669 (67)	0.65 (0.04)	47 (5)	1.8 (0.5)	37
Band 1	3.0	850 (149)	0.53 (0.01)	62 (1)	2.7 (0.9)	20
		700 (28)	0.43 (0.01)	76 (1)	2.1 (0.7)	37

[a] The percentage of mobile population of cytochrome oxidase was calculated from Equation 5.

[b] Numbers in brackets are standard deviation.

[c] Mitoplasts incubated at pH 6.5 for 1 h at 30 °C then transferred to pH 7.4.

[d] Since the accuracy of the data in this case does not justify use of the full Equation 4 because of the small decay in $r(t)$, we employ the simplified equation $r(t) = [r(0) - r_3]exp(-t/\phi) + r_3$. Furthermore, in some preparations, no rapidly rotating cytochrome oxidase was detected and only slowly rotating oxidase with ϕ = 15 to 20 ms was observed.

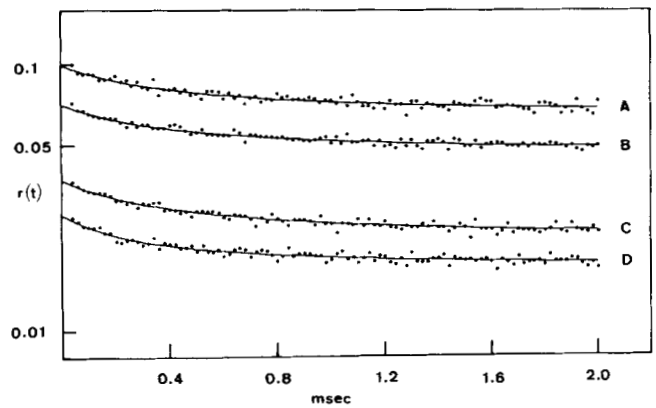

FIG. 5. **Effect of freezing and cytochrome *c* addition on the time-dependent absorption anisotropy of cytochrome oxidase in Band 2 at 37 °C.** Samples contain 2 μM heme a. Experimental conditions are the same as described in Fig. 3. Curves are *A*, frozen-thawed Band 2; *B*, the same preparation as *A* without freezing (control for *curve A*); *C*, frozen-thawed Band 2 containing exogenous 30 μM cytochrome *c*; *D*, the same preparation as *C* without exogeneous cytochrome *c* (control for *curve C*). The Band 2 membranes for *curves A, C, D* were frozen in liquid nitrogen, stored at −80 °C and then thawed for $r(t)$ measurements. *Curves B, C, D* have been displaced vertically for clarity.

procedures. Since almost all rotation measurements for lipid-enriched inner mitochondrial membranes were performed without supplementing cytochrome *c*, the effect of adding cytochrome *c* was examined separately. We observed no significant difference in $r(t)$ within experimental error between the presence and absence of supplemented cytochrome *c* (see Fig. 5). This is consistent with the finding that the addition of cytochrome *c* does not affect the mobility of cytochrome oxidase in proteoliposomes containing both cytochrome bc_1 complex and cytochrome oxidase (5).

Rebinding Kinetics of CO with Reduced Heme a_3

The rebinding of CO to reduced heme a_3 after photolysis showed monoexponential kinetics in mitochondria, mitoplasts, Pellet, and Bands 1 to 3. The time constant, τ, of this reaction was 10 to 13 ms in 60% sucrose for all membranes examined. No significant change in τ was observed between 20 °C and 37 °C within experimental error. The above τ values were quite similar to those of bovine heart cytochrome oxidase ($\tau = 10 \pm 1$ ms) in mitochondrial inner membranes and reconstituted proteoliposomes in 60% sucrose at 20 °C (5, 6).

DISCUSSION

Rotationally Immobile Cytochrome Oxidase—Co-existence of mobile and immobile cytochrome oxidase has been shown for several mitochondrial membranes, *i.e.* beef heart (6), rat heart (6), and rat liver and also for *Paracoccus denitrificans*.[2] The existence of mobile oxidase supports the idea of lateral collision-controlled electron transfer between cytochrome bc_1 and cytochrome oxidase through cytochrome *c*, because it was shown that at least several collisions among these redox components are possible within the known rate of electron transfer (5). However, the significance of the 50 to 80% of cytochrome oxidase which is rotationally immobile in mitochondria remained to be elucidated.

There are several reasons for supposing that the immobile cytochrome oxidase exists in nonspecific aggregates with other membrane proteins. Such aggregates would be a consequence of the low L/P in mitochondrial inner membranes and may involve hydrophobic association. First, it is likely that such aggregates would have low affinity and would dissociate upon dilution, in accord with the present observation of the effect of phospholipid enrichment on cytochrome oxidase mobility. Freeze-fracture pictures support this view (see Fig. 1), because almost no large protein aggregates were observed in Band 1, implying that protein aggregates in the native (nonfused) inner membranes are dissociated by phospholipid enrichment. Second, the co-existence of mobile and immobile (or less mobile) populations is not a special property of cytochrome oxidase but has now been observed with various other proteins both in native and reconstituted membranes (10, 12, 27). Of particular relevance is the finding that another protein of the mitochondrial inner membrane, the ADP/ATP translocator, also is present as co-existing mobile and immobile populations (28).

The alternative explanation that cytochrome oxidase forms specific aggregates with other proteins appear less probable. Such specific complexes would most likely be of high affinity and thus would not dissociate upon lipid enrichment. Moreover, complex formation between the most likely candidate, cytochrome bc_1, and cytochrome oxidase was not detected in a reconstituted system (5).

There appears to be a small increase in the mobility of cytochrome oxidase in going from mitochondria to mitoplasts. As L/P is virtually unchanged within the inner membrane of mitochondria, this could be due to the digitonin treatment which might somewhat disturb the organization of membrane proteins. The decrease in the mobile population of the oxidase in Pellet after the fusion procedures was shown to be due to incubation of membranes at pH 6.5, since a similar decrease was observed upon pH 6.5 incubation in the absence of fusion.

Local Lateral Diffusion of Respiratory Proteins—Electron transfer between respiratory proteins does not require long range lateral diffusion but can be controlled by local lateral collisions, because the mitochondrial inner membranes are densely packed with integral proteins (29–31). Both rotational and lateral diffusion of proteins are Brownian thermal motion, and there is a relationship between these motions (32):

$$D_L^{\text{loc}} \simeq (\ln \eta/\eta' - \gamma)a^2/\phi_\parallel \qquad (6)$$

where D_L^{loc} is the local (free) lateral diffusion coefficient, η is the membrane viscosity, η' is the viscosity of the aqueous phase, γ is the Euler's constant and a is the radius of the membrane-immersed part of protein. Although the measured ϕ is not an exact $\phi_\parallel$, it probably represents an average value over different rotating species of cytochrome oxidase. In Band 1, we may estimate a ~100 Å for the average radius of membrane particles from freeze-fracture pictures. If we take a ≃100 Å, $\eta \simeq 400$ centipoise (11), $\phi \simeq 700$ μs and $\eta' = 25$ centipoise (60% sucrose at 37 °C), we obtain $D_L^{\text{loc}} \simeq 3 \times 10^{-9}$ cm²/s for the mobile oxidase. This value is in reasonable agreement with D_L^{loc} of cytochrome oxidase in phospholipid vesicles (5).

Free lateral diffusion of molecules in the membrane is very insensitive to the size of the molecule. In fact even 100-fold difference in the molecular weight can change the diffusion coefficient by a factor less than 10 (33). The long range lateral diffusion coefficient for integral proteins collectively in the inner membrane of mitochondria was found to be $\sim 10^{-9}$ cm²/s by electrophoretic displacement and randomization (34). Thus, it appears that integral redox proteins (regardless of whether they are rotating or in microaggregates) laterally diffuse with $D_L^{\text{loc}} \sim 10^{-9}$ cm²/s.

Mechanisms of Electron Transfer in the Respiratory Chain—The observed decrease in rate of electron transfer from NADH dehydrogenase, succinate dehydrogenase and

[2] S. Kawato and B. Ludwig, unpublished work.

cytochrome bc_1 complex to molecular oxygen with increase in average distance between integral proteins is in good agreement with previous reports (4). Since newly incorporated phospholipid was shown not to inhibit the intrinsic electron transfer capacity of specific proteins (3, 4), it appears likely that lateral collisions plays an essential role for electron transfer in the whole respiratory chain.

However, not all redox components undergo free lateral diffusion, because nonspecific protein aggregates are deduced to be present in the inner membrane of mitochondria due to the very high concentration of proteins. In such protein microaggregates, other studies suggest that electron transfer between redox components may also occur at rates comparable to those of freely diffusing components (1, 35). Incorporation of 10 to 20% cholesterol (mol/mol) with phospholipid into the inner membrane resulted in recovery of the decrease in electron transfer activity which occurs after the incorporation of phospholipid alone (35). Since cholesterol was shown to segregate protein rich domains from lipid rich domains to form protein microaggregates (1, 35, 36), the above result suggests that the efficiency of electron transfer was not decreased by the presence of such protein aggregates. The expected high mobility of the peripheral protein cytochrome *c*, the abundance of ubiquinone and possible close contact of redox enzymes could explain a reasonably high electron transfer rate in protein microaggregates (5, 37–41). This is consistent with the measured decreased kinetics after enriching lipid into the mitochondrial inner membrane. If the dissociation of protein aggregates does not in itself significantly increase the electron transfer activity, the lipid enrichment simply decreases the rate of electron transfer by increasing the average distance between freely diffusing redox components.

CONCLUSION

The combination of protein rotation measurements with phospholipid enrichment in the inner membrane of mitochondria revealed the following properties: 1) The fraction of rotating cytochrome oxidase is a function of L/P in the membrane. 2) The rotationally immobile cytochrome oxidase appears to be in nonspecific protein aggregates in the membrane. 3) The effect of varying L/P on electron transfer activities is mainly similar to that observed by Schneider *et al.* (4). It is, however, noted that a reproducible difference in protein distribution occurred in the different membrane fractions obtained from the fusion procedures. 4) Electron transport probably occurs within microaggregates in addition to collision-controlled reactions between independently diffusing redox components.

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