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Fluidity in mitochondrial membranes: Thermotropic lateral translational motion of intramembrane particles

(freeze fracture/membrane structure/integral proteins/lipid-protein phase separations)

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ABSTRACT **The fracture faces of frozen rat liver mitochondria reveal that intramembrane particles can be induced to undergo long-range lateral translational motion and aggregation, which parallel the appearance of large, particle-free smooth patches in the hydrophobic interior of the two mitochondrial membranes. These lateral separations were observed under conditions that induce thermotropic lipid-lipid phase separations. Low temperature-induced lateral separation occurred between the intramembrane particles (integral proteins) and smooth patches (bilayer lipid) at temperatures between about 10 and −12° in the outer membrane and between about −4 and −12° in the inner, energy transducing membrane. Complete reversibility of particle aggregation occurred rapidly by free lateral diffusion, whereas oxidative phosphorylation remained relatively intact after withdrawal of the low temperature perturbation. These data suggest that (*i*) lipid-protein lateral phase separations can occur in both mitochondrial membranes, and (*ii*) the bilayer lipid of both membranes is a fluid two-dimensional continuum that imparts freedom of lateral translational motion to the integral proteins of these membranes.**

Intramembrane particles, as revealed by freeze-fracture electron microscopy, appear to represent integral proteins that intercalate into the hydrophobic bilayer lipid of cell membranes. Studies have shown that intramembrane particles can be induced by various nonphysiological perturbations to undergo long-range lateral translational motion in the plane of certain cell membranes (reviewed in ref. 1). It has been determined that the integral proteins rhodopsin, glycophorin, and microsomal ATPase not only appear as definite intramembrane particles when reconstituted in vesicles of synthetic phospholipids, but can undergo low temperature-induced long-range lateral translational motion in the phospholipid bilayer (2–4). Such observations support the concept that the bilayer lipid of natural and reconstituted biomembranes is fluid and represents a solvent through which integral proteins can diffuse laterally (5).

Although the intramembrane particles in mitochondrial membranes may show a limited degree of freedom of motion during change in mitochondrial metabolic state (6, 7), no data have emerged that would indicate that intramembrane particles and/or integral proteins can be induced to undergo long-range lateral translational motion in these membranes. Of special interest in this regard is the energy transducing inner membrane. On the one hand, limitation of lateral translational motion of the integral proteins may be inferred from data that suggest that the proteins of the inner membrane are stabilized through a semi-rigid protein lattice (8–10). This concept is consistent with the requirement for protein-protein interaction during such rapid metabolic events as electron transport and energy transduction during the oxidative synthesis of ATP. On the other hand, several findings can be interpreted to imply that the inner membrane of the mitochondrion may be fluid, allowing lateral diffusion of integral proteins in the lipid bilayer. The membrane undergoes rapid, reversible configurational changes during change in metabolic state (11, 12). The membrane also shows structural disorder in its lateral plane, at least with regard to the distribution of cytochrome *c* oxidase (13). In addition, reversible thermotropic phase transitions have been shown to occur in the lipids of mitochondrial membranes (14, 15).

In this communication we present observations that reveal that intramembrane particles can undergo long-range lateral translational motion and aggregation of different magnitude in the two mitochondrial membranes under conditions that induce lipid-lipid phase separations.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of male Sprague–Dawley rats using 0.25 M sucrose at 0° (16). Protein was determined by a biuret method (17). Electron transport was measured as a function of oxygen consumption by polarographic means (18). ADP:O and acceptor control ratios were calculated according to the procedures outlined by Estabrook (19). For freeze fracturing, freshly isolated mitochondria (10 mg of protein) were incubated at room temperature for 5 min in 0.5 ml of 0.25 M sucrose containing 30% glycerol buffered at pH 7.4 with 10 mM Tris·HCl. In a limited number of experiments, glycerol was replaced by ethylene glycol or cryoprotectors were eliminated. The mitochondria were then pelleted at room temperature at 15,000 × *g* for 2 min in 400-μl polyethylene tubes in a Beckman Microfuge 125. The pelleted samples were equilibrated at 30, 10, 0, −4, −8, or −12°, transferred to gold-nickel specimen holders equilibrated to corresponding temperatures, and then rapidly frozen in Freon 22 that had been cooled by liquid nitrogen. For reversibility studies, specimens equilibrated at −8 and −12° were returned quickly to 30° and then rapidly frozen. Glutaraldehyde-fixed mitochondria were also studied. Mitochondria were equilibrated at 30° in suspension in the sucrose-glycerol-Tris medium. Samples were then fixed by addition of 25% glutaraldehyde to a final concentration of 0.2%, centrifuged as above, and rapidly frozen from 30°. Freeze fracturing was carried out in a Balzers BA360 freeze-etching apparatus equipped with electron guns. Electron micrographs were taken with a Philips 300 electron microscope operated at 80 kV.

RESULTS

Structure of Mitochondrial Membranes at 30°. When freshly isolated mitochondria were fixed with 0.2% glutaraldehyde at 30° prior to rapid freezing, intramembrane particles were observed to be randomly dispersed in a smooth continuum in the fracture faces of both membranes (Fig. 1A). Consistent with our previous observations (6, 7), the size distribution of the intramembrane particles showed a peak at 8 nm in the outer

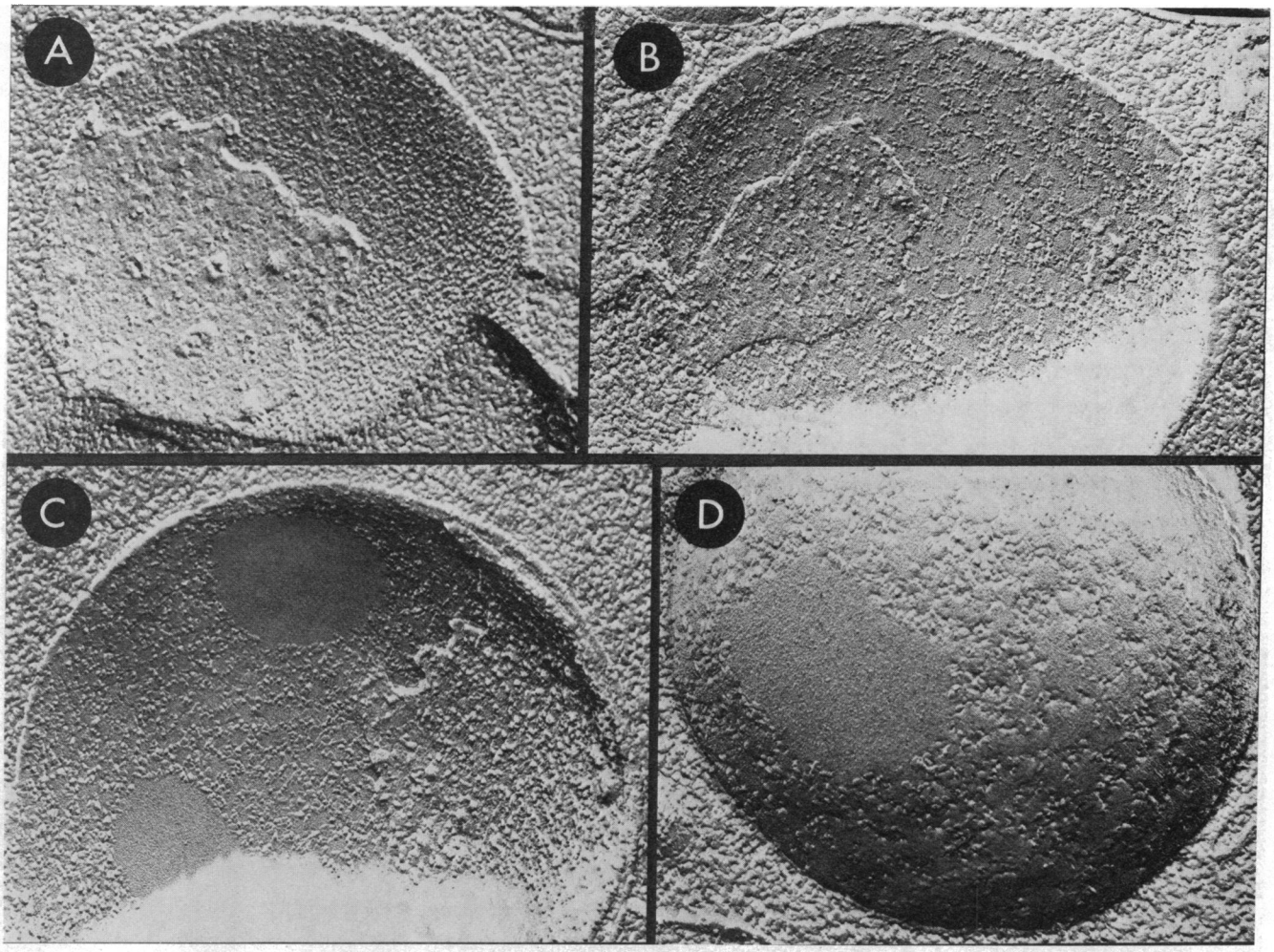

FIG. 1. Fracture faces of mitochondrial membranes, ×67,500. (A) Concave fracture faces of glutaraldehyde-fixed inner and outer membranes rapidly frozen from 30°. (B) Concave fracture faces of inner and outer membranes rapidly frozen from 30°. (C) Concave fracture faces of inner and outer membranes equilibrated at 0° then rapidly frozen. (D) Convex fracture faces of outer membrane equilibrated at 0° then rapidly frozen.

and 10 nm in the inner membrane. This size difference is a reliable means for distinguishing between the two membranes (Fig. 1A). Freshly isolated mitochondria rapidly frozen from 30° without prior glutaraldehyde fixation showed a slight lateral separation between the intramembrane particles and smooth continuum in the fracture face of the outer membrane (Fig. 1B). Consequently, the intramembrane particles appeared in hexagonal-like arrays bordering small smooth patches in the hydrophobic interior of the membrane. The distribution of intramembrane particles in the unfixed inner membrane appeared identical to the distribution observed in the fixed inner membrane. These findings led us to infer that a slight lipid-lipid phase separation, appearing as small smooth patches in the fracture face, occurred in the unfixed outer membrane during the short time interval of the rapid freezing procedure when the temperature dropped from 30° to −150° and induced a small degree of lateral translational displacement of the intramembrane particles.

Structure of Mitochondrial Membranes at 10° and 0°. After considering the observations above, it was of interest to examine the structure of unfixed mitochondrial membranes equilibrated at temperatures below 30° prior to rapid freezing. When frozen from 10°, the small smooth patches in the fracture face of the outer membrane were slightly enlarged compared to preparations frozen from 30°. When frozen from 0°, exceptionally large smooth patches were observed in the outer membrane along with a clear indication of lateral translational displacement and aggregation of intramembrane particles (Fig. 1C). These enlarged smooth patches in the outer membrane at 0° were presumably the result of an increase in solid lipid of the solid ⇄ fluid lipid phase equilibrium of the membrane and accounted for the lateral translational motion of intramembrane particles. The intramembrane particles in the outer half leaflet of the outer membrane (concave fracture face; Fig. 1C), as well as the particle's complementary pits in the inner half leaflet (convex fracture face; Fig. 1D), exhibited the same degree of lateral motion. No lateral separations occurred in the inner membrane at 10° or 0° (Fig. 1C).

Structure of Mitochondrial Membranes at Temperatures between −4 and −12°. To induce increases in lipid-lipid phase separation, we equilibrated unfixed mitochondria at −4°, −8°, and −12° prior to rapid freezing. Below 0° a further lateral separation between intramembrane particles and smooth patches occurred in the outer membrane (Fig. 2A and B). At −8° and −12° both half leaflets of the inner membrane showed pronounced lateral separations between intramembrane particles and smooth patches (Fig. 2A and B). These lateral separations were observed to occur equally in the inner boundary and cristal membrane portions of the inner membrane.

The lateral separations described above were completely reversible. When mitochondria were equilibrated at temperatures between −4° and −12° and subsequently warmed to 30°

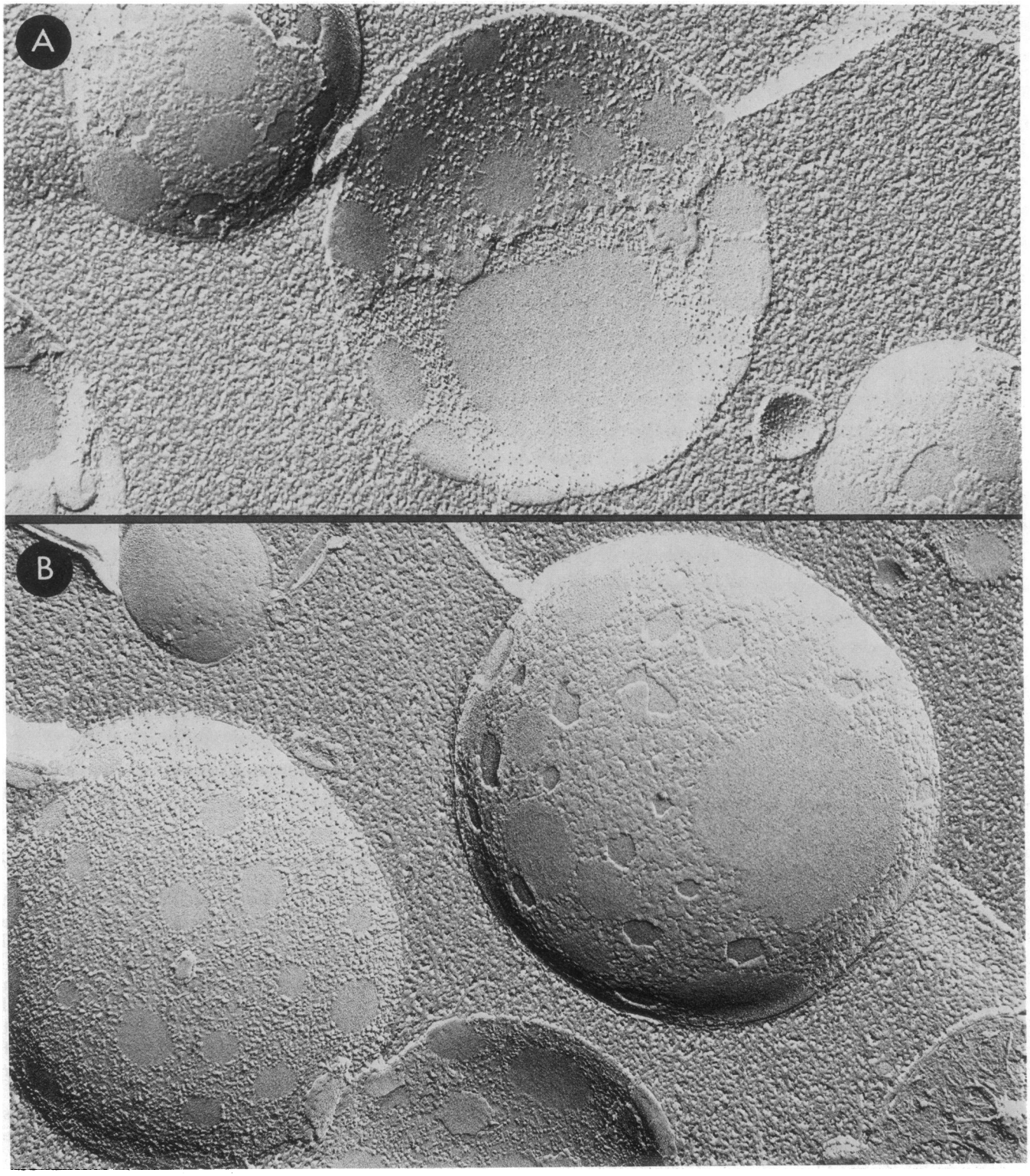

FIG. 2. Fracture faces of mitochondrial membranes, ×67,500. (A) Concave fracture faces of inner and outer membranes (center) equilibrated at −8° then rapidly frozen. (B) Convex fracture faces of inner membrane (left), convex fracture faces of outer membrane (right), and concave fracture faces of outer membrane (lower) equilibrated at −8° and then rapidly frozen.

for only a few seconds prior to freezing, the distribution of intramembrane particles appeared identical to that shown in Fig. 1B of mitochondrial membranes frozen directly from 30°. Thus, free lateral translational diffusion with complete randomization of intramembrane particles occurred within seconds after the withdrawal of the low temperature perturbation.

A consistent structural difference noted between the two membranes was that the inner half leaflet of the outer membrane primarily revealed pits, whereas the inner half leaflet of the inner membrane revealed particles (Fig. 2B). This structural distinction may reflect a fundamental difference in the lipid-protein association of the two membranes. Thermotropic lateral

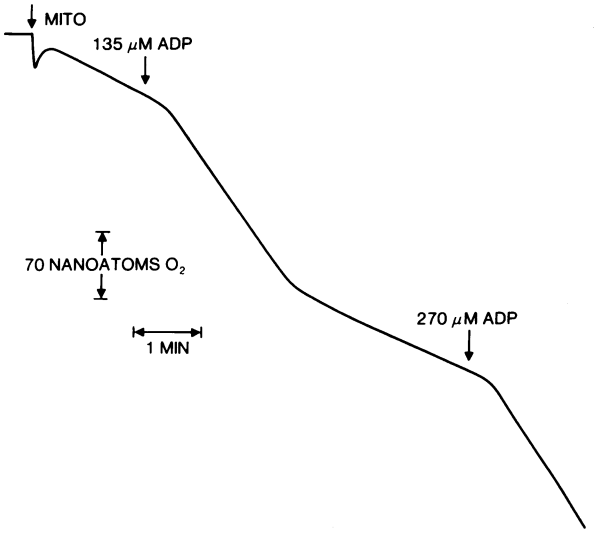

FIG. 3. Oxidative phosphorylation in mitochondria after low temperature-induced lateral separations in the inner membrane. Prior to their addition to the reaction system, the mitochondria were equilibrated in 0.25 M sucrose containing 30% glycerol buffered at pH 7.4 with 10 mM Tris·HCl for 8 min at −8°. Oxygen concentration was measured polarographically. Reaction system: sucrose (0.150 M); sodium phosphate buffer (0.01 M, pH 7.4); $MgCl_2$ (5.0 mM); succinate (10 mM); bovine-serum albumin (2 mg); glycerol (30%); mitochondria (2 mg of protein). Total volume, 2 ml; temperature 25°.

separations were best observed when the membranes were protected from ice crystal damage with glycerol. However, even in ethylene glycol, the separations occurred at characteristic temperatures similar to those found in the presence of glycerol. Although ice crystal damage was considerable without the use of any cryoprotectors, lateral separation could be observed in the outer membrane between 10° and 0°. The inner membrane could not be studied without cryoprotectors since here lateral separation occurs below −4°. Under no circumstances did the addition of cryoprotectors cause particle aggregation. These observations indicate that the cryoprotector of choice, glycerol, did not contribute significantly to the potential for the thermotropic lateral separations found in the mitochondrial membranes.

It was determined that the thermotropic lateral separations that occurred in the inner membrane at temperatures below −4° did not destroy electron transport or oxidative phosphorylation (Fig. 3). Mitochondria equilibrated at −8° in glycerol medium for as long as 15 min were as efficient in oxidative phosphorylation as mitochondria in glycerol, which were not induced to undergo inner membrane thermotropic lateral separations (Table 1).

Table 1. ADP:O and acceptor control (A.C.) ratios in mitochondria before and after low temperature-induced lateral separations in the inner membrane

Exp.	Pretreatment with glycerol medium*: °C	No. of min	ADP:O	A.C.
1	No pretreatment		1.80	5.50
2	25	2	1.48	3.38
3	25	2	1.45	3.33
4	0	8	1.50	3.19
5	0	8	1.59	3.05
6	−8	8	1.42	3.43
7	−8	8	1.48	3.00
8	−8	15	1.56	3.05

* Mitochondria were treated with glycerol medium (250 mM sucrose, 30% glycerol, 10 mM Tris·HCl at pH 7.4) at temperatures and times indicated prior to polarographic analysis. Reaction system as in Fig. 3, minus glycerol in Exp. 1.

DISCUSSION

We have determined that the intramembrane particles of mitochondria can be thermally induced to undergo reversible long-range lateral translational motion without destroying such metabolically significant activities as electron transport and oxidative phosphorylation. Our results suggest that lipid-protein lateral phase separations can occur in both mitochondrial membranes and that both membranes are fluid. The thermotropic lateral separations observed can be attributed to a succession of physical events beginning with fluid ⇄ solid (disordered ⇄ ordered) phase transitions in the bilayer lipid of the mitochondrial membranes. Low temperature-induced phase transitions in the complex lipid mixtures of mitochondrial membranes can be expected to result in lipid-lipid phase separations with various lipids freezing out in the bilayer lipid as solid phase patches of specific lipid composition.

That thermotropic lipid-lipid phase separations occurred under our experimental conditions was indicated by the increasing size and number of particle-free, smooth patches that appeared in the membrane fracture faces with decreasing temperatures. Provided the temperature was decreased gradually, it could be expected that nonanchored integral proteins of the membranes would be excluded from the expanding solid phase bilayer lipid and become aggregated, presumably along with their boundary lipids, into the remaining liquid phase of the bilayer lipid. Such lipid-protein phase separations were indicated by the progressive lateral translational motion and aggregation of intramembrane particles observed in the membrane fracture faces. Of particular interest was the finding that free lateral translational diffusion and complete randomization of aggregated intramembrane particles occurred rapidly in both membranes with removal of the low temperature perturbation. Similar changes have been reported to occur in several natural and reconstituted membranes (2–4, 20–23).

In mitochondrial membranes most of the integral proteins can be accounted for as metabolic, oligomeric proteins. Since the smallest known subunits of these proteins are approximately 10,000 in molecular weight (24, 25), most of the oligomeric proteins can be expected to appear as intramembrane particles in freeze fracture faces. Thus, after thermotropic lateral separations the aggregated particles are equated with the integral protein-rich regions of the membranes, whereas the particle-poor, smooth patches are equated with the protein-poor, lipid-rich regions of the membranes.

Since mitochondrial membranes contain a complex mixture of lipids, the onset-to-completion of lipid-lipid phase separations occurs over a rather broad temperature range. The first suggestion of low temperature-induced lateral separation between intramembrane particles and the smooth continuum of mitochondrial membranes occurred at 10° but was restricted to the outer membrane. Not until the temperature was lowered to less than −4° did the fracture faces of the inner membrane clearly reveal enlarged smooth patches and lateral translational motion of intramembrane particles. On the basis of these observations, we suggest that the onset of the thermotropic lipid-lipid phase separation in the bilayer lipid of the inner

membrane occurs at a significantly lower temperature than the lipid-lipid phase separation in the outer membrane. We suggest further that the low temperature-induced onset-to-completion of the lipid-lipid phase separation occurs over a broader temperature range in the outer membrane (about 10 to −12°) compared to the inner membrane (about −4 to −12°).

These differences must relate ultimately to the distinct compositions of the two membranes. The inner membrane is composed of 75% protein, whereas the outer membrane is 63% protein (26, 27). The high content of integral protein in the inner membrane (about 50% of the membrane protein; refs. 25 and 28), may immobilize up to 30% of the membrane lipid as boundary lipid (29, 30). Irrespective of this high protein content, our observations suggest that the bilayer lipid of the inner membrane is quite fluid, and we would surmise that the difference in protein content in the two membranes does not account for the difference in thermotropic structural events exhibited by these membranes. More clearly related to our findings is the distinct phospholipid composition of the two membranes. Unsaturated phospholipids show lower phase transition temperatures than saturated phospholipids (31). The four major phospholipids in rat liver mitochondria are significantly more unsaturated in the inner membrane than in the outer membrane (26, 27). From the data of Colbeau *et al.* (27) we calculated a ratio for the saturated to unsaturated lipids at 0.65 for the inner and 1.75 for the outer membrane. Phosphatidylcholine and phosphatidylethanolamine together comprise 80% of the total phospholipids in both membranes and are collectively 53% unsaturated in the inner and 45% unsaturated in the outer membrane. Cardiolipin, which comprises 20% of the phospholipid of the inner membrane, is approximately 90% unsaturated. In addition, phosphatidylinositol accounts for 9% of the outer membrane and is 65% saturated. Cardiolipin and phosphatidylinositol therefore may play predominately significant roles in the differences in the two membranes with regard to the onset and completion of temperature-induced lateral translational motion of integral proteins.

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