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Calcium-mediated fusion to produce ultra large osmotically active mitochondrial inner membranes of controlled protein density

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We have developed a new membrane fusion method which produces ultra large, spherical mitochondrial inner membranes attached to microscope slides. The fused inner membranes measured up to 200 μm in diameter. The technique fuses native inner membranes as well as inner membranes in which the protein density has been varied by enriching with exogenous phospholipid. The fusion process is accomplished through the use of calcium, low pH and elevated temperature. Characterization of the fused membranes was carried out using phase, fluorescence, and freeze-fracture electron microscopy. These ultra large, fused inner membranes were found to model the inner membranes from which they were formed. The fused inner membranes were found to be osmotically active and are large enough for measuring the lateral diffusion of membrane components by fluorescence recovery after photobleaching and are large enough for microelectrode impalement.

Introduction

The ability to vary parameters such as integral protein density (i.e., lipid/protein ratio), lipid composition and membrane fluidity, in a controlled fashion by the incorporation of exogenous phospholipid into native intact membranes is a useful approach to study lateral diffusion of membrane components as well as lipid-protein interactions [1–10]. Such an approach has produced data from this laboratory which indicate that the mitochondrial electron transport 'chain' of the inner membrane is actually a sequentially interactive pool of randomly distributed oxidation-reduction (redox) components whose catalytic rates are coupled to their rates of lateral diffusion and collision frequency in the membrane plane [2,3]. Kinetic studies of various segments of the electron transport sequence after incorporation of exogenous phospholipids revealed that as the lipid/protein and lipid/heme *a* ratios increased, the catalytic rates correspondingly decreased, suggesting that the redox components are independent membrane diffusants [5]. Recently the lateral diffusion coefficients for a number of redox components have been determined directly using the technique of fluorescence recovery after photobleaching [11,12]. This technique requires inner membranes having a minimum 5 μm diameter, which is significantly

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; diI, 3,3'-dioctyldecylindocarbocyanine.

larger than the typical diameter of 1.5 μm for rat liver mitochondrial inner membranes after hypotonic swelling.

The mitochondrial inner membrane has a composition which is consistent with an important role for lateral diffusion. The inner membrane is highly 'fluid' (for reviews see Refs. 13, 15, 16 and 17). Although the amount of protein associated with this membrane is very high, i.e., approximately 75% [17,18], only half of it is integral to the bilayer proper [2,17,19] and the total membrane protein occupies less than one-half of the membrane surface area [20]. Thus sufficient area is available for two-dimensional lateral diffusion of the redox components in this membrane. It can be expected that lipid-protein and protein-protein interactions during lateral diffusion of redox components are significant in the overall rate of electron transport.

To study the lateral diffusion of components in the mitochondrial inner membrane, it would be useful to prepare inner membranes both large enough for the fluorescence recovery after photobleaching technique and with various concentrations of redox components. This would permit the assessment of the effects of the two-dimensional concentration of redox components on their diffusion and relate this to electron transfer kinetics as well as an assessment of the effect of lipid-protein interactions. To prepare such membranes we report here a new calcium fusion method. This procedure fuses native mitochondrial inner membranes or phospholipid-enriched mitochondrial inner membranes into large diameter (up to 200 μm) spherical inner membranes attached to microscope slides. These ultra large membranes are osmotically active and preliminary results indicate that they can be 'energized' comparable to intact mitochondria.

Methods and Materials

Preparation of mitochondrial inner membrane

Mitochondria were isolated from the livers of male Sprague-Dawley rats as described previously [1,21]. A controlled digitonin incubation was used to remove the outer membrane, leaving an intact inner membrane-matrix (mitoplast) fraction [22,23]. The inner membrane-matrix was subsequently converted to a simple spherical configuration by hypotonic swelling in a 40 mosM buffer [22]. This buffer was designated H_{40}. Membranes so treated were designated IMM_{40}.

Enrichment of inner membranes with phospholipid

The inner membrane enrichment process was carried out using small unilamellar vesicles of soybean phospholipid by the method of Schneider et al. [1,4]. The phospholipid composition of soybean phospholipid [24] is similar to that of the mitochondrial inner membrane [25]. The pH at which the enrichment was done was 6.35. Four density distinct inner membrane fractions were isolated [1] with protein concentrations ranging from 8 to 10 mg protein per ml in the 30% phospholipid enriched inner membranes to approx. 1 mg/ml in the 700% enriched membranes.

Calcium fusion of the enriched inner membranes

$CaCl_2$ was dissolved in deionized, distilled water containing 5 mM Hepes as the buffering agent. Hepes was used since it was present in the membrane isolation media. The pH of the solution was adjusted to the desired pH with KOH. For most applications, the stock solution of choice was 20 mM $CaCl_2$ buffered at pH 6.5.

The calcium fusion procedure was initiated by adding the calcium solution to a specific membrane suspension, volume for volume in microliter quantities while thoroughly mixing. The resultant solution was then transferred by pipette to the surface of an uncoated glass microscope slide. Two small strips of weighing paper were placed at two ends of the sample area to form a bridge, which provided an approx. 40 μl volume when a 22 mm square coverslip was placed on top. The two edges parallel to the strips of weighing paper were completely sealed with paraffin. The other two edges were sealed partially with paraffin to allow access to the chamber. The slide was then placed on a heating block thermostated to 37°C in a humidified environment. The calcium fusion was allowed to proceed 15 min at this temperature and then the slide was removed and allowed to cool to room temperature.

The incorporation of the fluorescent molecule diI into inner membranes was used to assess homogeneity in the membrane bilayer after calcium fusion. The probe, dissolved in absolute ethanol,

was added to inner membrane suspensions prior to calcium fusion with gentle mixing to give a probe to phospholipid ratio between 1 : 1000 and 1 : 10000 and an ethanol concentration < 1%.

Freeze-fracture electron microscopy

For freeze-fracture electron microscopy, the calcium-fused, enriched inner membranes were prepared as described above, but as concentrated as possible. The higher membrane concentration was achieved by adding a smaller volume of a more concentrated calcium solution (e.g., 200 mM) to a correspondingly larger volume of the membrane suspension. After the calcium fusion was completed, the paraffin and the coverslip were gently removed and the membrane-containing solution was transferred to a solution of 50% glycerol, a cyro-protectant, in H_{40} buffer. After 2 min the aliquots of membrane samples were placed on Balzers gold specimen holders. Rapid freezing was carried out by emersion in liquid propane cooled with liquid nitrogen. Freeze fracturing was carried out in a modified Balzers BA 360 instrument equipped with electron guns [26]. Electron micrographs were taken in a JEOL 100 CX side entry or a JEOL 100 CX top entry electron microscope operated at 80 kV.

Phase-contrast and fluorescence microscopy

Light microscopy was performed on a Zeiss WL microscope equipped for phase contrast with halogen illumination and mercury illumination for epi fluorescence or an Olympus Vanox microscope with temperature stage and similar light source. Photography was performed with an Olympus OM-2N in the automatic exposure mode using Kodak Ektachrome ASA 400 slide film.

Materials and reagents

All chemicals were of reagent grade. Asolectin (soybean phospholipids) was obtained from Associated Concentrates. DiI was purchased from Molecular Probes and dissolved in absolute ethanol.

Results

Calcium-mediated fusion method

The calcium fusion procedure detailed in Methods and Materials permitted the fusion of mitochondrial inner membranes or the fusion of phospholipid-enriched inner membranes into ultra large inner membranes. The conditions of pH 6.5, final calcium concentration of 10 mM and 37°C for 15 min were determined to be the optimal conditions.

Operationally the total membrane fusion process can be divided on the basis of microscopic observation into two major steps: (1) aggregation of the membranes and (2) the actual fusion. Studies were carried out that independently varied pH, calcium concentration and temperature, and may be summarized as follows: Over the range of pH (5.00 to 7.00) and calcium (0.5 mM to 100 mM), inner membrane aggregation and fusion occurred to varying degrees. Membrane aggregation was drastically reduced and fused membranes were rarely detected at the lowest calcium concentrations (i.e., 0.5–1.0 mM). However, the use of lower pH values (i.e., 5.00–5.75) at lower calcium concentrations improved the yield of aggregated and subsequently fused membranes to a small degree. As the pH was increased above 6.5 or the final calcium concentration above 10 mM, the amount of fused vesicles progressively decreased. High calcium concentrations (> 10 mM) always promoted strong membrane aggregation but little fusion. At room temperature, the addition of an appropriate concentration of calcium chloride buffered at pH 6.5 to the membrane suspension resulted immediately in large, packed membrane aggregates, but did not result in membrane fusion. Membrane suspensions in the presence of 10 mM calcium left at room temperature for long periods (24 to 72 h) exhibited a few fused membranes. Exposure of the aggregates to elevated temperature (37°C) for as little time as 15 min was required to promote the actual fusion process. Aggregation and fusion did not occur with the phospholipid-enriched membranes heated in the absence of calcium, whereas only aggregation occurred for native inner membranes.

Light microscopy observations

Observations of membranes by dark field or phase contrast after the addition of calcium on a microscope temperature stage at 37°C showed the increase in diameter of the membranes once fusion

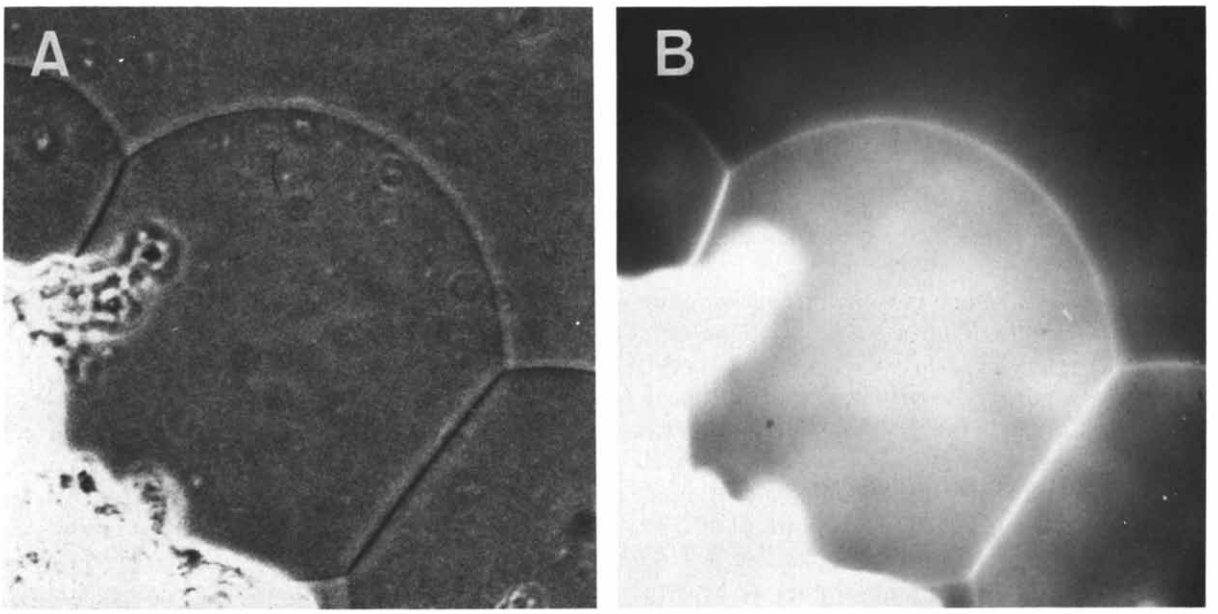

Fig. 1. (A) Phase-contrast image of 30% asolectin enriched mitochondrial inner membranes fused at pH 6.5, final calcium concentration of 10 mM, 37°C for 15 min. (B) Fluorescent image of the membranes in (A) due to the excitation of dil which was incorporated into the membrane bilayer prior to calcium fusion at a probe to phospholipid ratio of 1 : 10000. Contributions of out of focus fluorescence are accentuated by the black and white photographic process. Figures are at 980× magnification.

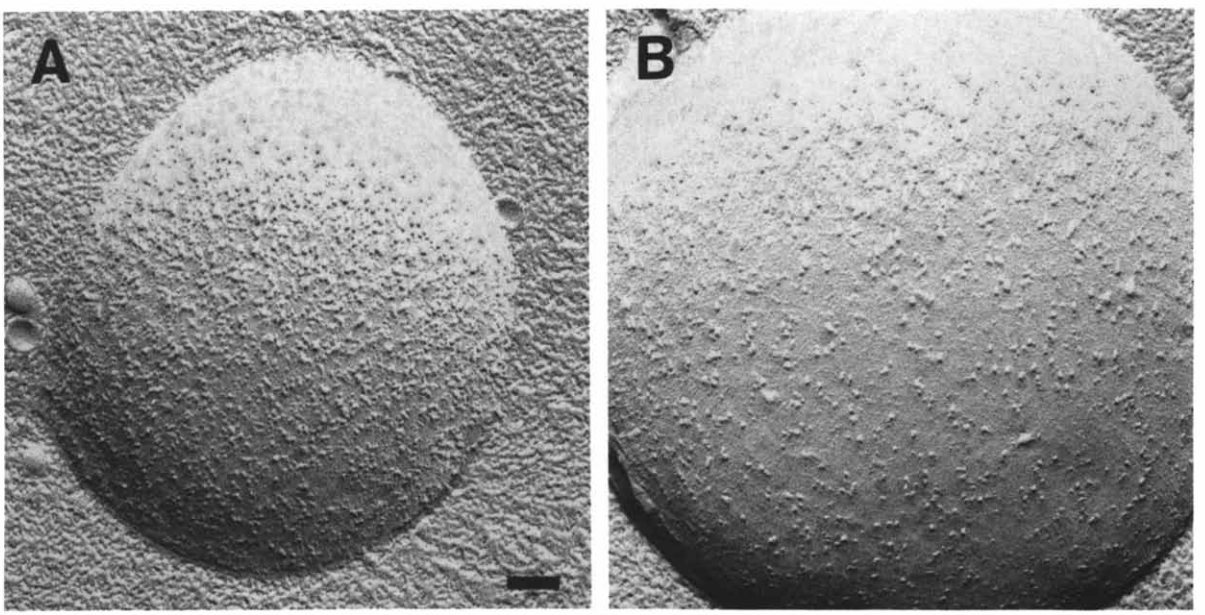

Fig. 2. Freeze-fracture electron micrographs at 64000× magnification. The intramembrane particles correspond to integral membrane proteins. (A) Freeze-fracture face of a 30% asolectin enriched inner membrane (as control). (B) Freeze-fracture face of a 240% asolectin enriched inner membrane (as control). Bar = 0.1 μm.

was initiated. Typically the fused membranes were seen to develop at the edge of the calcium-induced aggregates. Some of the large, fused membranes broke away from the aggregates as intact, spherical, independent membranes. Many other fused membranes came into close contact without in turn fusing into a single structure.

Fused membranes varied from a few microns in diameter up to 200 μm (or more) with the majority approximately 20 to 50 μm in diameter. Fig. 1A shows calcium-fused, phospholipid-enriched mitochondrial inner membranes as they appear under phase contrast. Their normal spherical shape has been deformed due to their close apposition.

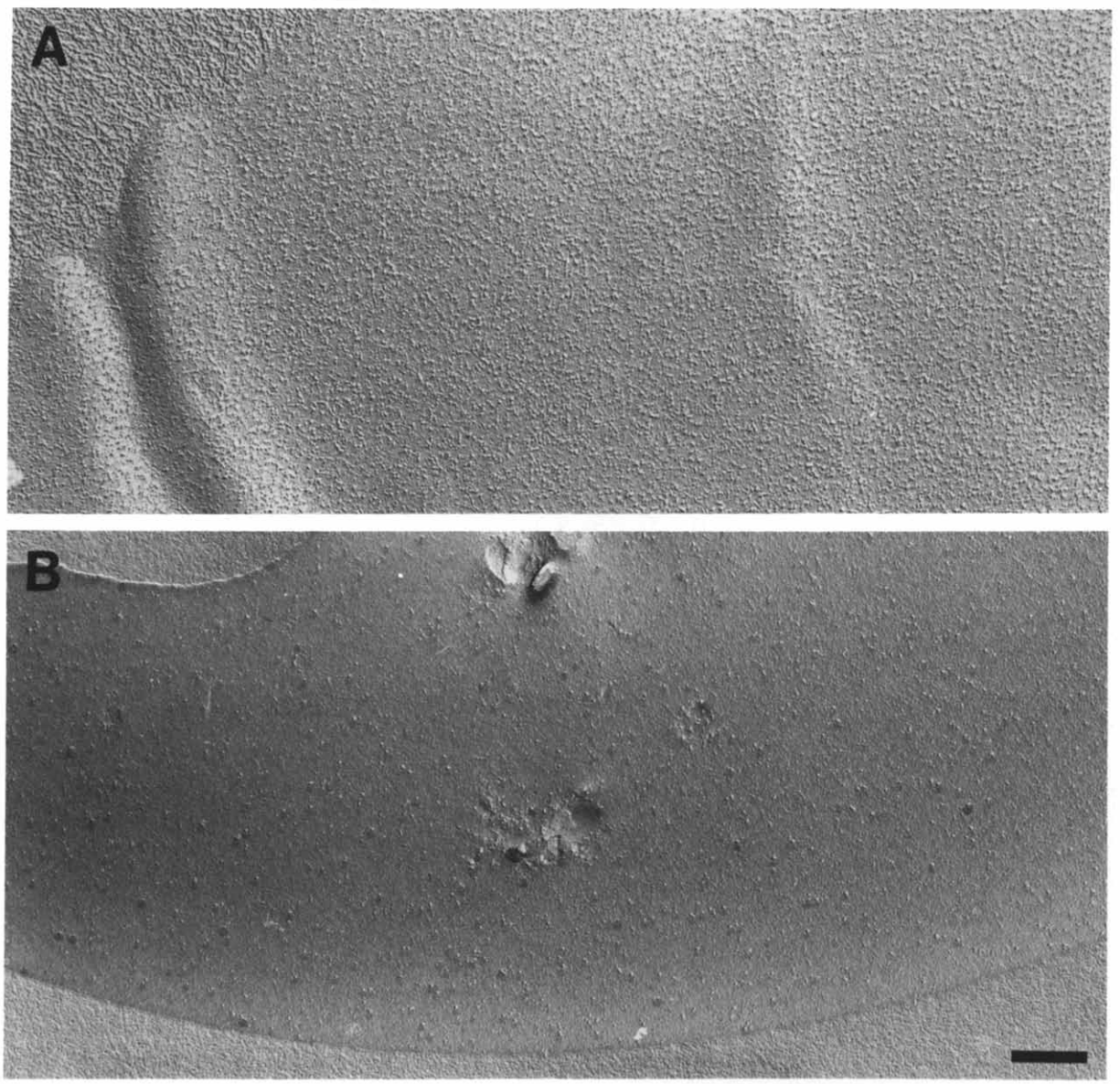

Fig. 3. Freeze-fracture electron micrographs at 35000× magnification. Fusion conditions as in Fig. 1. (A) Part of a freeze-fracture face of a calcium-fused, 30% asolectin enriched mitochondrial inner membrane having a minimum 20 μm diameter. (B) Part of a freeze-fracture face of a calcium-fused, 240% asolectin enriched inner membrane having a minimum 40 μm diameter. Bar = 0.3 μm.

The contact surfaces of these membranes always showed a thickened boundary which represented the two independent, intact membranes. The ultra large fused membranes did not attach directly to the glass substratum, but were usually anchored to membrane aggregates which were in turn immobilized on the glass. Fusion was also carried out on a hydrocarbon (wax) substratum, indicating that the glass surface was not necessary for the fusion or aggregation processes.

Fluorescent labels were successfully incorporated into the membranes. Fig. 1B shows the fluorescent image of fused membranes due to the incorporation of the lipid analogue diI. The homogeneous fluorescent intensity of the incorporated label suggested that no permanent large lateral phase separations were induced by the calcium-fusion procedure.

Freeze-fracture electron microscopy, analysis of protein density

Results of the freeze-fracture analysis are shown in Figs. 2 and 3. Comparison of the membrane freeze fracture faces in Figs. 2A and 2B (30% and 240% phospholipid enrichment, respectively) in the absence of calcium fusion shows the decrease in the protein density (number of particles per unit surface area) with increasing enrichment (see also Ref. 1). The freeze-fracture electron micrograph in Fig. 3A shows a fracture face of a large calcium fused, 30% phospholipid-enriched mitochondrial inner membrane. It was determined that the protein density after calcium fusion (Fig. 3A) was in very close agreement with the corresponding non-calcium fused, phospholipid-enriched membranes (Fig. 2A). Fig. 3B is a freeze-fracture electron micrograph of a calcium fused, 240% phospholipid-enriched inner membrane demonstrating a random distribution of proteins with a density corresponding to that for the non-calcium fused phospholipid-enriched membrane (Fig. 2B). Similar results were also obtained for the 80% and 700% phospholipid-enriched mitochondrial inner membranes.

Osmotic effects on calcium-fused membranes

One of the salient features of the native and phospholipid-enriched inner membranes fused by this particular calcium fusion procedure is that they were osmotically active. A sequence of phase contrast images of a calcium-fused, 30% phospholipid-enriched mitochondrial inner membrane in Figs. 4A through 4C shows the reversible sequence of osmotic sensitivity. The addition of a high osmolarity (i.e., 700 mosM) stock sucrose solution to the slide chamber induced hypotonic shrinking of the fused membranes giving rise to an irregular shape (Fig. 4B). Subsequent washing with 40 mosM buffer, in which the stock membranes are stored initially, restored the fused membranes to their normal spherical configuration (Fig. 4C).

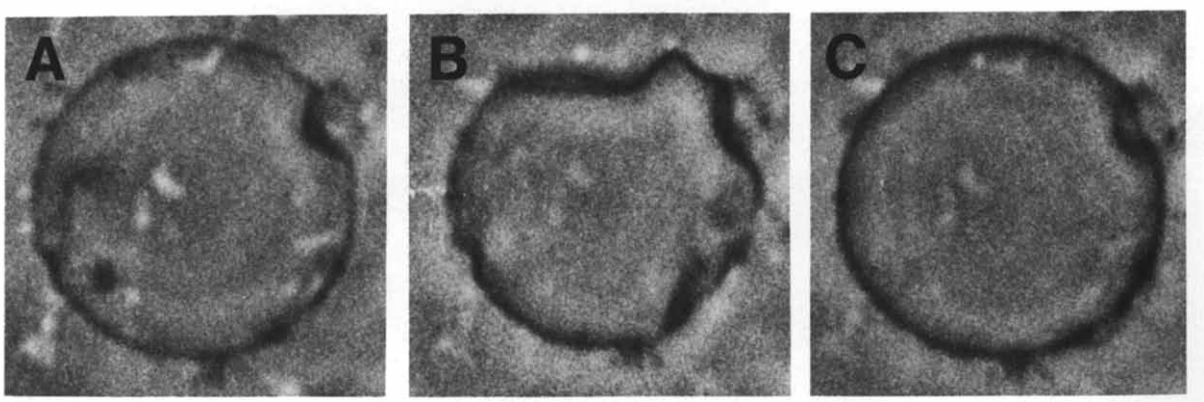

Fig. 4. Phase-contrast images of a spherical calcium-fused, 30% asolectin enriched inner membrane demonstrating osmotic sensitivity. Fusion conditions as in Fig. 1. (A) Membrane under initial conditions in 40 mosM buffer (H_{40}). (B) Membrane after addition of a high osmolarity (i.e. 700 mosM) stock sucrose solution to the slide chamber. Rapid crenation of membrane occurs upon addition. Image represents maximum effect selected from a rapid sequence of photographs of the process. (C) Membrane after washing in 40 mosM buffer returns to original spherical shape. A–C, magnification 2236×.

Discussion

The conditions of pH 6.5, final calcium concentration of 10 mM, 37°C for 15 min were determined to be the optimal conditions for fusion of rat liver mitochondrial inner membranes. The temperature (37°C) at which the inner membranes were incubated in the presence of calcium was selected to be as high as possible without giving rise to irreversible thermal denaturation of membrane enzymes. This was done by comparison to other enzyme kinetic temperature studies [27]. Prolonged incubation (1–2 h) at 50–60°C resulted in aggregation of native mitochondrial inner membranes and clustering of intramembrane particles therein; whereas, in the phospholipid-enriched inner membranes clustering of the inner membrane particles occurred, but not aggregation of the membranes. The difference in the surface adhesion properties of these two membrane types is most likely due to the altered lipid composition of the enriched membranes. The maximum pH was dictated by the decrease in fusion as the pH was raised. It was considered desirable in terms of enzyme stability not to use a very low pH. The calcium concentration used was defined by the efficiency of the fusion process. At the higher (> 10 mM) and lower (< 1.0 mM) calcium concentrations, the yield of fused membranes decreased significantly. It is apparent that the fusion of these membranes does not occur at one critical set of conditions; rather, fusion occurs over a range of conditions that has an optimum. This finding is consistent with other studies on membrane fusion [28–32] and with analysis of fusion events [30,31], where fusion was found to occur over a range of conditions.

It was concluded from freeze-fracture electron microscopic analysis that there were no discernable effects of the calcium-fusion procedure at optimum conditions on the structural integrity of the fused membranes. This conclusion was based on the fact that there were no gross morphological changes, and intramembrane particles were present and randomly distributed in all the calcium-fused membranes examined. It was also established that the fusion of the lipid enriched inner membranes having a defined range of intramembrane particle densities produced ultra large fused inner membranes having the same particle density as the average of the enriched membrane population.

Previously, work in our laboratory has demonstrated that the protein distribution in the native and phospholipid-enriched mitochondrial inner membrane is random [1,2,14,15,33,34]. However, incorporation of certain components (e.g., cholesterol) can induce lateral phase separations between lipid and protein components [4]. Lipid-protein phase separations did not appear to occur with the calcium-fusion procedure. While unequivocal conclusions cannot be made about the distribution of different phospholipid classes (viz. formation of 'microdomains'), the fluorescent intensity of the dil molecules incorporated into the inner membrane remained uniform after membrane fusion, indicating that there were no large lateral phase separations of the membrane lipids.

The alteration in the permeability of the calcium fused membranes as evidenced by the development of osmotic sensitivity was unexpected. The phospholipid-enriched inner membranes prepared by the low pH method of Schneider et al. [1] are not osmotically active. Apparently some change in the phospholipid enriched and native membranes is affected by the calcium and elevated temperature to render the fused membranes impermeable. Erythrocyte ghosts are commonly 'sealed' by incubation in NaCl at 37°C for 1 h [35], so it is not a totally unique phenomenon for the permeability of a membrane to decrease as a result of higher ion concentration and increased temperature.

Studies are in progress to determine the existence of a membrane potential via the use of fluorescent dyes. Preliminary studies with rhodamine 123 indicated that the calcium-fused inner membranes can be energized with succinate (increased fluorescence) and deenergized with CCCP. This is important since the size of these fused membranes makes them amenable to microelectrode studies.

Although our interest in developing this procedure was utilitarian, i.e., to obtain large inner membranes of varying protein densities for diffusion studies with the fluorescence recovery after photobleaching technique, and was not directed at elucidating a mechanism for fusion, it is of interest to compare our observations with other studies on

membrane fusion which has been the subject of a substantial number of reviews (e.g., Refs. 28–32). We have utilized calcium (10 mM), low pH (6.5), and elevated temperature (37°C) as an optimal condition to fuse mitochondrial inner membranes. Each of these three parameters has been used independently to induce fusion in different membranes.

For fusion to occur, membranes must come into close proximity in some manner and then be transiently destabilized. Negatively charged lipids have been implicated in fusion mediated by divalent cations such as calcium [28,29]. Cardiolipin may play a significant role in the fusion of the mitochondrial inner membranes since it is a major negatively charged phospholipid of this membrane [25]. Cardiolipin liposomes have been shown to undergo calcium-induced fusion [36–38]. Cardiolipin has also been shown in the presence of a positively charged species such as cytochrome *c* [39] or calcium, to form hexagonal II phase and some investigators believe this phase may be involved in the fusion process [37–39]. Related to these findings, we observed that when the cardiolipin content of the exogenous phospholipid was increased to approx. 20% as in the native mitochondrial inner membrane [25], the low pH phospholipid-enrichment process was much more efficient and the calcium-induced aggregation of the enriched membranes was also enhanced (unpublished results). A reduction of electrostatic repulsion between membrane surfaces can also be accomplished, albeit less efficiently, by the lowering of the bulk solution pH [30,31]. Träuble [40] has pointed out that the localized hydrogen ion concentration between two 'close' negatively charged membranes is greater than that for the bulk phase. For membranes that fuse by temperature changes alone, it has been suggested that an increase in temperature can enhance their close approach via the kinetic energy of Brownian motion and that the actual fusion process is due to a temperature caused instability in the membrane structure. Nir et al. [31] point out that since 'fusion' is composed of a number of discrete steps, each may have a temperature dependence.

In conclusion, the results presented in this paper demonstrate that native mitochondrial inner membranes or phospholipid-enriched mitochondrial inner membranes can be fused into osmotically active, ultra large spherical inner membranes utilizing calcium, low pH, and elevated temperature. The results further show that these fused membranes model the inner membranes from which they are produced. Such fused, enlarged inner membranes attached to microscope slides are useful in the measurement of lateral diffusion coefficients by the technique of fluorescence recovery after photobleaching, and can be potentially useful in microelectrode studies.

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