FINE STRUCTURE OF BEEF HEART MITOCHONDRIAL COMPLEX III

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1. Introduction

Complex III of the electron transfer system extracted from beef heart mitochondria was observed using both the negative stain and freeze fracture techniques of electron microscopy. The complex appeared as a spherical particle with an average diameter of 75 Å. The calculated mol. wt of this particle was found to be 177 000. The complex is readily incorporated into phospholipid vesicles prepared from polar inner membrane phospholipids and appears to be partially embedded in the phospholipid bilayer. Fracture surfaces of phospholipid vesicles into which Complex III was incorporated show numerous particles of about the same size as the negative stained complex.

The complexes of the electron transfer system can be readily isolated from the inner mitochondrial membrane using detergents [1]. In a previous communication [2] cytochrome oxidase, Complex IV, was examined using high resolution electron microscopy. Using the freeze fracture technique, the complex appeared as a prolate spheroid 135 Å × 68 Å, and the mol. wt of 244 000 was determined.

In this communication we wish to show the size and shape of Complex III before and after incorporation into phospholipid vesicles prepared from the polar phospholipids of the inner membrane of beef heart mitochondria using both the negative stain and freeze fracture techniques of electron microscopy.

2. Materials and methods

Beef heart mitochondria were prepared by the method of Hatefi and Lester [3] and stored frozen until used. Complex III was isolated by the method of Rieske [4] from beef heart mitochondria and stored in 1 ml aliquots in sealed ampoules (11 mg/ml) under liquid nitrogen. Inner mitochondrial membrane polar phospholipids were extracted from ETPH [5], an inner mitochondrial membrane preparation, by the method of Rouser and Fleischer [6] and stored under argon in sealed ampoules in liquid nitrogen. Phospholipid vesicles containing Complex III were prepared as follows: inner membrane polar phospholipids (4.6 μmol of phosphorous) in 2:1 chloroform-methanol was evaporated to dryness under a gentle stream of argon. Eleven mg of Complex III suspended in 2 ml of 0.1 M sodium phosphate (pH 7.2) was added to the phospholipids and mixed by homogenization. Sodium cholate (20% w/v) was added until a total concentration of 5% cholate was attained. The clear red suspension was briefly homogenized in a teflon-glass homogenizer and dialyzed 24 h at 4°C against

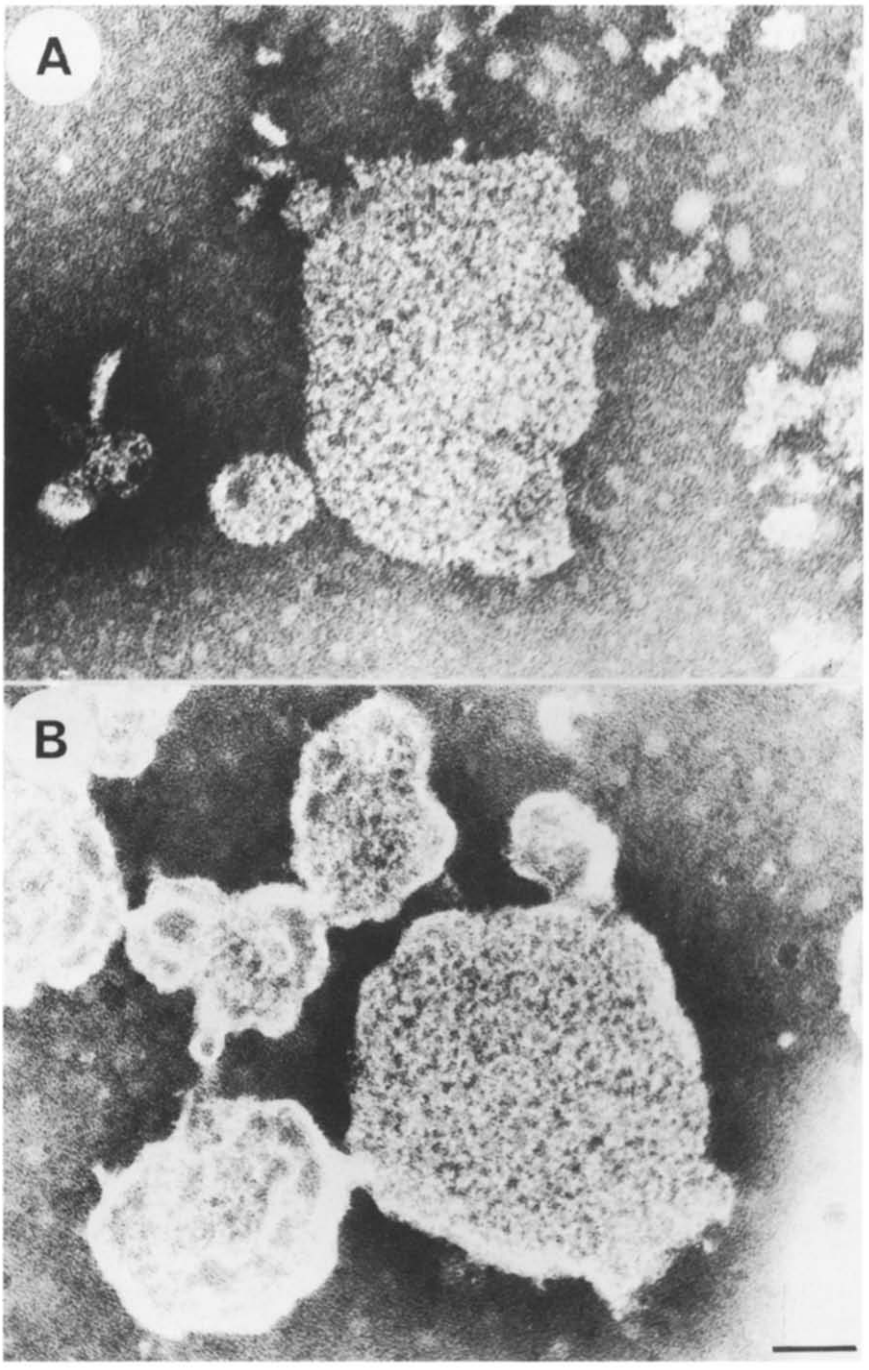

Fig.1. Negative stained preparations of Complex III. Total magnification is 200 000 ×. Bar is 500 Å. Fig.1A, Complex III before the addition of phospholipids. Numerous 75 Å spherical particles appear associated as various size aggregates. Fig.1B; Complex III incorporated into phospholipid vesicles.

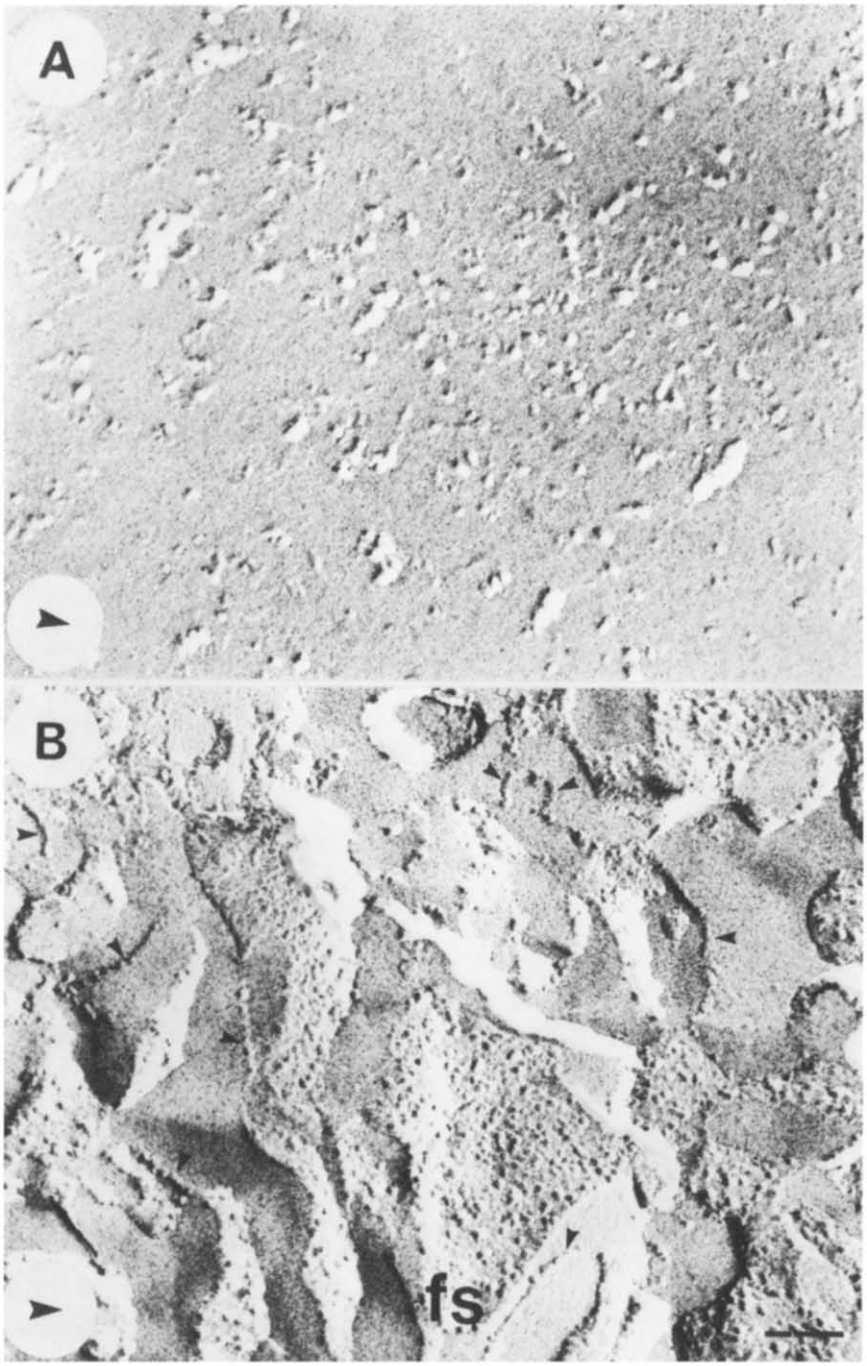

Fig.2. Freeze fracture of Complex III. Total magnification is 100 000 ×. Bar is 1000 Å. Fig.2A;Complex III before the addition of phospholipids. Individual particles appear as well as aggregates, Fig.2B; Complex III incorporated into phospholipid vesicles. Numerous particles appear on the fracture surfaces of the vesicle sheet.

4 changes of 500 vol each of 0.1 M sodium phosphate buffer. The vesicles were sedimented at 198 000 *g* for 30 min. Protein was determined by the biuret procedure. Phosphorous was determined by the method of Chen et al. [7] and phospholipid was estimated assuming a mol. wt of 775.

For negative stains, one drop of the suspension (0.5 to 1.0 mg of protein per ml) was added to 1000 mesh carbon coated grids previously treated with 0.01% w/v of bacitracin to ensure the complete wetting of the grid. A drop of the suspension was applied to the grid, the excess drawn off, and stained with 1% w/v potassium phosphotungstate, pH 7.2.

For freeze fractures, samples were washed with 30% v/v aqueous glycerol and sedimented at 198 000 *g* for 30 min. Aliquots of the pellets (approx. 10 μl) were placed in gold cups, quickly frozen in Freon 22, and placed on a pre-cooled stage of a Balzer Freeze Etch apparatus (BA360). Fractures were performed at -115°C at 2×10^{-6} Torr, shadowed with Pt-C, and replicated with C. Replicas were washed for 60 min in commercial sodium hypochlorite solution (Javex), washed twice with water and mounted on bare 75 × 300 mesh copper grids. All specimens were observed in a Philips EM300 electron microscope operated at either 60 or 80 kV using a liquid nitrogen anticontamination device. In all freeze fracture electron micrographs, an arrow in the lower left hand corner indicates the direction of the platinum deposit.

3. Results

The negative stain technique was used to observe Complex III before and after its incorporation into phospholipid vesicles. Fig.1 shows the results of these experiments. Fig.1A shows an aggregate of the complex before the addition of phospholipid. The individual particles appear as spheres of an average diameter of 75 Å. Many of these particles are visible since the electron dense negative stain outlines the particle.

Fig.1B shows the complex after its incorporation into phospholipid vesicles. The surfaces of the vesicles show particles which appear smaller than the complex before incorporation, and the particles are much less distinct in appearance. This type of surface would be expected if the complex was partially partitioned in the phospholipid bilayer. Thus the negative stain would lie on the surface of the bilayer and surround the particles and only the tops of particles were unstained. The vesicles were composed of 31% phospholipid by weight.

Fig.2 shows the freeze fracture replica before (fig.2A) and after (fig.2B) incorporation into phospholipid vesicles. Fig.2A shows Complex III, and many particles appear to be associated in aggregates while others appear as individual particles. The size of the largest particles appear approx. 100–110 Å. Correcting for an estimated C-Pt deposit of 25–35 Å, they correspond in size as those of the negative stain preparation.

Fig.2B shows the complex after incorporation in phospholipid vesicles. Many particulate fracture surfaces (fs) of membranes can be seen as well as cross fractures of the vesicles (at arrows). From cross fractures, it appears that the particles are embedded partially in the membrane and do not appear to transverse the membrane such as Complex IV, cytochrome oxidase. A higher magnification of the complex after incorporation is shown in fig.3 and shows a large particulate fracture surface (fs) as well as cross fracture (at arrows).

4. Discussion

The negative stain and freeze fracture techniques of electron microscopy can be used to examine membrane proteins. In particular, the freeze fracture technique shows large fracture surface areas as well as cross fractures. The cross fractures of membranes can be likened to the trilaminar cuts of membranes in thin sections. However the freeze fracture technique allows one to visualize the orientation of the proteins within the membrane and qualitative as well as quantitative measurements of protein can be made.

The negative stain technique gives a 2-dimensional representation of the surface of membranes and, because of the nature of the technique, one observes desiccated samples embedded in an electron dense stain. However, the freeze fracture technique gives a 3-dimensional representation of hydrated specimens. Both techniques would, therefore, compliment themselves. Although the exact fracture plane of the membrane is yet unknown, Branton [8] suggests that

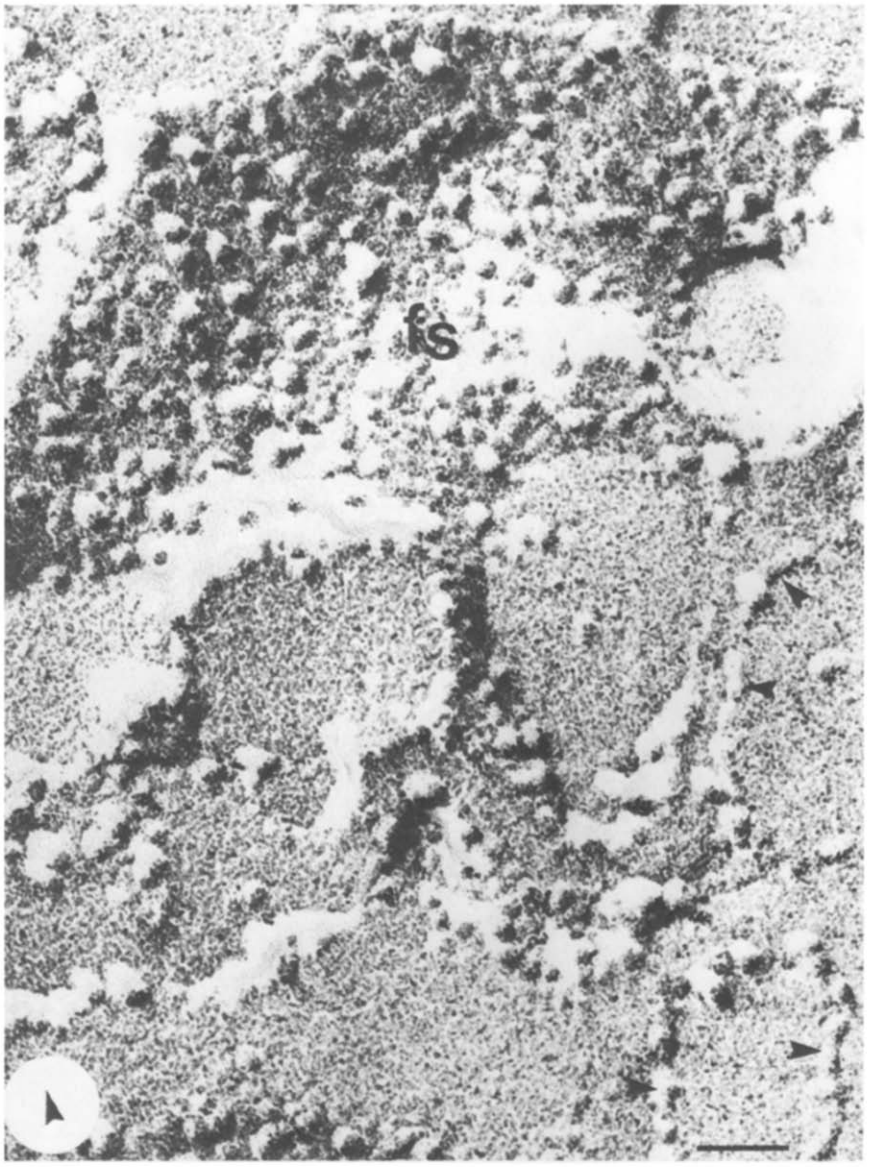

Fig. 3. High magnification of Complex III incorporated into phospholipid. A large concave surface is visible and numerous cross fractures. Total magnification 250 000 ×. Bar is 500 Å.

the fracture passes through the hydrophobic interior of the membrane.

From both negative stain and freeze fracture data, we have determined the size and shape of Complex III. The complex appears as a spherical molecule of an average diameter of 75 Å. The mol. wt was calculated from the size to be 177 000 assuming a density of 1.33. After the complex was incorporated into a polar phospholipid preparation from the inner of beef heart mitochondria, the particles appear much smaller and rather indistinct, and further separated from one another, although the centre to centre distance of the

particles still remains 80–100 Å. This observation suggests that the complex is partially embedded in the phospholipid bilayer and the negative stain, lying on the bilayers, fills around the particle to show only the tops of the complex.

Freeze fracture data show particles in both cross fractures as well as extensive particulate fracture surfaces. If indeed the fracture passes through the hydrophobic interior of the membrane, the particles present on the fracture surfaces represent the complex embedded in the hydrophobic interior of the membrane. If the Complex merely binds to the outside surface of the phospholipid vesicle, one would not be able to observe the Complex since the fracture surfaces would be smooth and devoid of particles.

This study as well as a previous study [2], shows that Complex III and Complex IV, cytochrome oxidase, differs morphologically from one another so that they should be able to be distinguished from one another in the intact inner mitochondrial membrane. The freeze fracture technique could be used to identify the complexes of electron transfer system in the membrane once the size and shape relationships are established for the other 2 complexes. Presently work is being carried out to examine these other 2 complexes.

Acknowledgement

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