THE STRUCTURE OF CYTOCHROME OXIDASE MEMBRANES

William J. VAIL and R.K. RILEY*
Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario, Canada

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1. Introduction

Two preparations of oxidized membranous cytochrome oxidase were examined using both the negative stain and freeze fracture techniques of electron microscopy. The cholate preparation, containing 27% phospholipid by weight, appeared as membrane sheets. With the freeze fracture technique, cross fractures of the sheets showed particles abutted one against another. The Triton preparation, containing 42% phospholipid by weight, was decidedly vesicular and only particulate membrane fracture surfaces were usually seen. In the case of membranous cytochrome oxidase it is proposed that fractures follow the surface rather than a centre plane through the hydrophobic interior of the membrane.

The observed particles appeared as prolate spheroids of 135 Å × 68 Å with the long axis extended through the membrane. From the size shape relationships, a molecular weight of 244 000 was calculated which is in agreement with values reported for dispersed cytochrome oxidase using other physical techniques. At least 50% of the protein is exposed to the aqueous environment.

Cytochrome oxidase is a lipoprotein that spontaneously forms membranes under conditions of low ionic strength and the absence of detergents. Sun et al. [1] showed that these membranes were trilaminate structures 55 Å thick when examined by conventional thin section techniques. Negatively stained preparations showed a membrane surface covered with 50 Å particles. Seki and Oda [2] have shown that these particles are 80–90 Å in diameter. We have used the negative stain and freeze fracture techniques to examine the subunit structure of membranous cytochrome oxidase.

2. Materials and methods

Beef heart mitochondria were prepared by the method of Hatefi and Lester [3] and stored frozen until used. The cholate preparation of cytochrome oxidase was prepared by the method of Fowler et al. [4]. The preparation had a heme *a* value of 8.05 nmoles/mg protein as determined by the method of Williams [5]. The Triton preparation was by the method of Sun et al. [1] with a heme *a* value of 8.2 nmoles/mg protein. Protein was determined by the method of Yonetani [6]. The cytochrome oxidase preparations used here were in the oxidized state. Phosphorus determinations were made by the method of Chen et al. [7], and phospholipids were estimated assuming an average molecular weight of 775. The cytochrome oxidase preparations were washed 3 times with 50 vol of 0.25 M sucrose–10 mM Tris–Cl, pH 7.4 immediately before use.

For freeze fractures, the cytochrome oxidase preparations were suspended in 10 vol of 30% v/v aqueous glycerol at 4°C and centrifuged for 60 min at 144 000 *g*. The pellet was washed twice with the glycerol solution and resuspended in glycerol at a protein concentration of approx. 100 mg/ml. Specimens were placed in gold cups, frozen in Freon 22, stored no longer than 5 min in liquid N_2, and placed on a 4 place specimen table of a Balzer BA 360 freeze etch apparatus precooled to −150° C. Fractures were performed at

* Present address: Department of Biology, Frostburg State College, Frostburg, Maryland, USA.

−100°C at 2 × 10^{-6} Torr; immediately shadowed with platinum-carbon; replicated with carbon; and replicas floated off on water. Replicas were cleaned by the method of Vail and Riley [8], and mounted on bare 75 × 300 mesh copper grids.

For negative stains, the glycerol treated preparations were diluted to a protein concentration of 1 mg/ml with 0.25 M sucrose 50 mM potassium cacodylate, pH 7.4 and fixed by adding a small aliquot of 50% w/v glutaraldehyde to a final glutaraldehyde concentration of 2%. Samples were negatively stained on lightly carboned 400 mesh copper grids with a solution of 1% w/v uranyl acetate. Other samples which were not glycerol treated as well as unfixed samples were observed to see if glycerol treatment had any effect on the membranes. We were unable to detect any differences in any of these samples. All specimens were observed in a Philips EM 300 electron microscope operated at 60 kV. For all freeze fractures an arrow in the lower left hand corner of the photo indicates the direction of the Pt deposit.

3. Results

The two preparations of cytochrome oxidase used in this study were both membranous but varied considerably in their phospholipid content. The cholate preparation contained 27% phospholipid by weight as compared to the Triton preparation whose phospholipid content was 42%. Thus it would be expected that the protein within the membrane would be more dispersed in the higher phospholipid containing membranes.

Fig. 1A shows a negative stain of the cholate preparation of cytochrome oxidase. Most of the membranes appear as flat sheets with 75 Å particles randomly embedded in the membrane continuum. The particle density however appears quite high.

The freeze fracture technique not only lends on added dimension to the 2 dimensional negative stain technique, but one views unfixed, hydrated membranes. Fig. 1B shows a low magnification of the cholate preparation of the freeze fracture technique. It appears that most membranes have been cross fractured to reveal the association of particles with one another while only an occasional fracture surface can be seen.

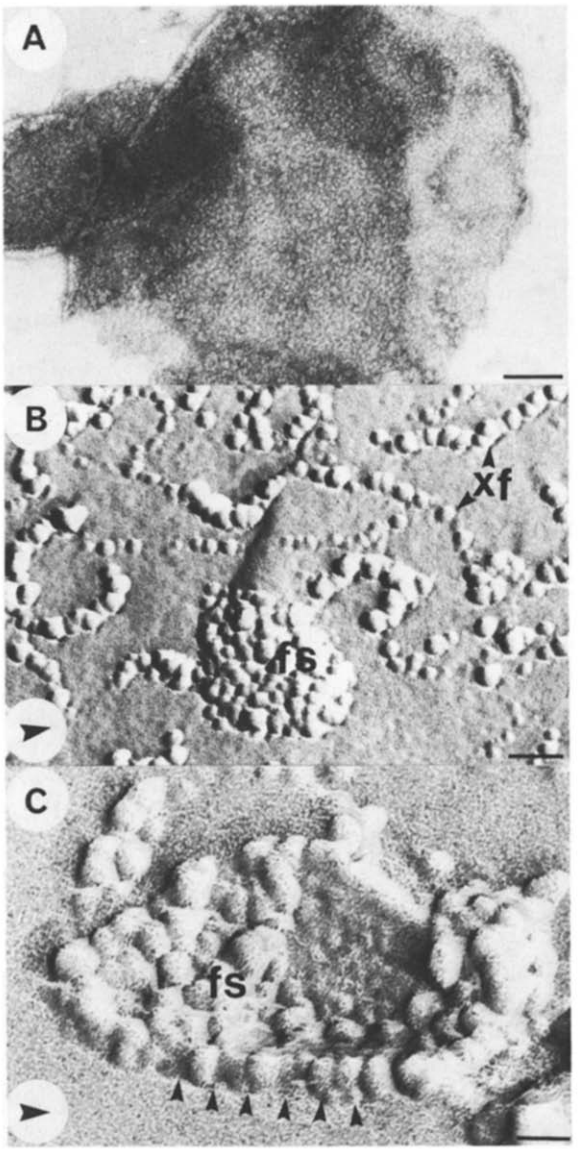

Fig. 1. Cholate preparation of cytochrome oxidase membranes. A. Negative stain of a membraneous sheet. Total magnification 120 000 ×. Bar is 0.1 μm. B. Freeze fracture replica showing both cross fractures (xf) and fracture surfaces (fs). Total magnification 108 700 ×. Bar is 0.1 μm. C. High magnification showing individual particles (at arrows) and a fracture surface (fs). Total magnification 210 000 ×. Bar is 500 Å.

In Fig. 1C a high magnification of an individual membrane can be seen in both cross fractures (at black arrows) and a membrane fracture surface (fs).

The individual particles, presumably proteins, can be clearly seen abutted one against another in cross fracture while the fracture surface shows the tops of the particles which one sees in negative stain preparations.

The Triton preparation of cytochrome oxidase was then examined using both the negative stain and freeze fracture techniques of electron microscopy. This preparation contained 42% phospholipid or 1.5 times that in the preceeding cholate preparation. Fig. 2A shows the negative stain preparation of vesicular membranes. Some of the membrane surfaces showed a crystalline lattice structure of particles first observed with a similar preparation [9]. About 7–10% of the membranes exhibited this crystallinity; it was curious that the crystallinity was usually observed only when membranes were stacked one upon another. In other membranes, the vesicles appeared to be particulate but no crystallinity was observed, and they appeared to have a lower particle density than that of the cholate preparations.

A low magnification of a freeze fracture clearly shows the vesicular nature of the preparations (fig. 2B). Fracture surfaces are commonly seen, however, occasional cross fractures are also observed. The cross fractures did now show a high density of particles abutted one against the other, as in the Fowler preparation.

At a higher magnification (fig. 2C), the fracture surfaces are clearly particulate with what appears as particles embedded in a smooth continuum. This vesicular membrane can be likened to a hollow sphere. If the fracture goes around the surface of the sphere, the outer convex surface exposed to the external medium can be seen. If however the sphere is broken, the inner concave surface is visualized. Convex (⌒) and concave (⌣) fracture surfaces appear to have a similar particle density of about 2 300 particles per square micron as an average of 100 determinations. It is suggested, therefore, that if the particle density of both concave and convex fracture surfaces are similar, the proteins might project entirely through the membrane. This observation conforms to the model of the membrane proposed by Vanderkooi et al. [10]. Since fracture surfaces are less commonly seen in the cholate preparations, the particle density was determined for only about 12 such fracture surfaces and 3 200 particles per square micron was calculated.

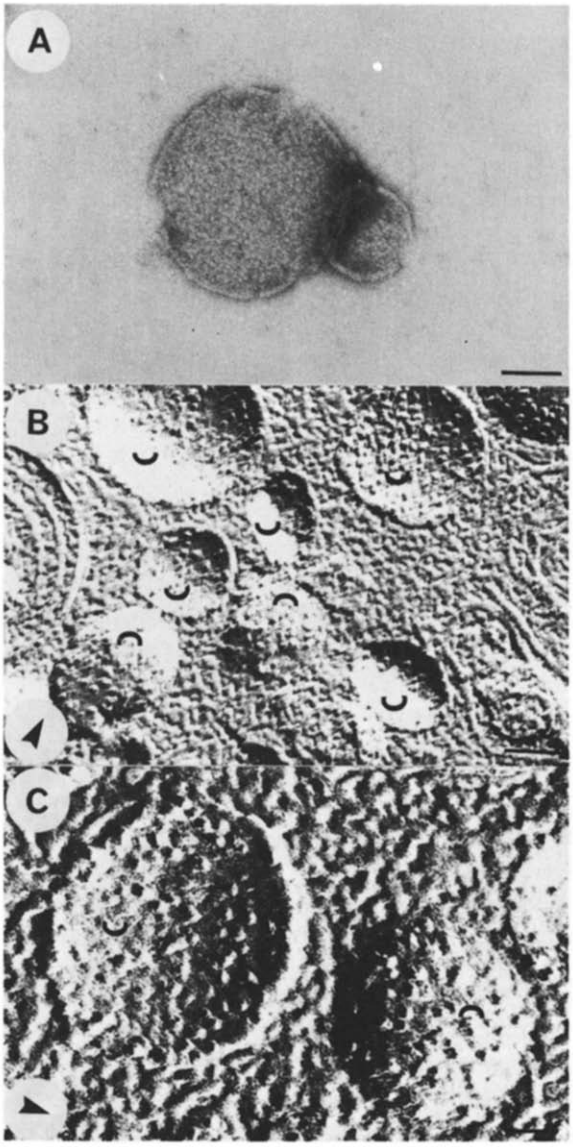

Fig. 2. A Triton preparation of cytochrome oxidase membranes. A. Negative stain a membrane vesicle. Total magnification 120 000 ×. Bar is 0.1 μm. B. Freeze fracture replica showing both concave (⌣) and convex (⌒) fracture surfaces of the vesicles. Total magnification 108 700 ×. Bar is 0.1 μm. C. High magnification showing the particle fracture surfaces of vesicles. Total magnification 210 000 ×. Bar is 500 A.

The crystal lattice structure which is clearly seen in the Triton preparation using the negative stain was never seen in the freeze fracture preparations nor was it ever observed in any cholate preparations. Perhaps

the 3-dimensional nature of concate and convex surfaces prevents the visualization of the lattice or the lattice could be due to the dehydration of the membrane.

To accurately determine the size of particles in freeze fracture replicas, the amount of platinum deposited on the replica must first be determined. We have incorporated ferritin as an internal standard in the cholate preparations of cytochrome oxidase sample. Thus, measuring ferritin near the membrane and subtracting the known size of the molecule, one can estimate the platinum deposit thickness with some degree of accuracy.

We have measured the size of the particle in the cytochrome oxidase membrane and corrected for the platinum deposit thickness. The particles appear as prolate spheroids of 135 Å by 68 Å whose long axis project through the membrane. Thus the volume of the particles can be calculated from the formula $V = \frac{4}{3} \pi a b^2$ where a is one half of the major axis and b is one half of the minor axis. The volume was calculated to be 3.27×10^{-19} cm^3. If one assumes the particles to be a lipoprotein of a density of 1.24, then the molecular weight can be estimated from the formula:

Molecular weight = $Na \times V \times \rho$ where Na is Advagadro's number, V is the volume in cm^3, and ρ is the density (1.24). The calculated molecular weight was 244 000.

If the hydrophobic region of the molecule estimated as being a cylinder which would encompass the thickness of a phospholipid bilayer or about 45 Å, then about 49% of the molecule would be hydrophobic while 51% would be hydrophilic. Capaldi [12] estimated that 60% of the molecule was exposed to the hydrophilic environment.

4. Discussion

The two preparations of membranous cytochrome oxidase examined had different configurations which corresponded to the phospholipid concentration in the membrane. The cholate preparation (27% lipid) appeared as membrane sheets, had a higher particle density, and cross fractures were usually observed. The Triton preparation (42% lipid) was vesicular in appearance, had a lower particle density, and fracture surfaces were commonly seen. Thus we have interpreted the particles as proteins which appear to transverse the phospholipid bilayer of the membrane. The presence of these proteins apparently prevents the splitting of membranes through the hydrophobic centre. The particle densities of the proteins were similar for both concave and convex fracture surfaces, and no small ridge was ever seen at the replica surface indicating such a split. The red blood cell plasma membrane has been shown to split through some centre plane [11, 12].

The visualization of proteins in 3 dimensions is an advantage of the freeze fracture technique. It also allows one to examine membrane proteins in greater clarity than conventional negative stain technique and both techniques compliment one another. However before the freeze fracture technique can be used for accurate size measurements, the amount of Pt evaporated on the particles must be determined. We have incorporated ferritin in the sample. The ferritin (110 Å) can be used as an internal standard when measured adjacent to the membrane. Thus, when one determines the Pt deposit, all measured values can be corrected for this deposit. We are not aware that the use of such an internal standard for freeze fracture has ever been reported.

The particle size of cytochrome oxidase was 135 Å × 68 Å and appears as a probate spheroid. The molecular weight of 244 000 corresponds to reports using other physical techniques [13–15]. Assuming 45 Å thickness for the phospholipid bilayer of the membrane, about half of the molecule is embedded in the bilayer and 25% exposed to the aqueous environment on both sides of the membrane. The model of Vanderkooi et al [10] seems to best describe the orientation of the proteins within the bilayer, however we are in variance with the estimated size of the individual particles. Capaldi [16] has estimated that 60% of the cytochrome oxidase molecule is in contact with the aqueous environment from deuterium-hydrogen exchange data which is not far from our estimation based on freeze fracture observations.

Acknowledgements

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