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The Morphology and Configurational States of Isolated Heavy Beef Heart Mitochondria by the Freeze Fracture Technique

William J. Vail and Robert K. Riley*

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada

and Charles H. Williams

The Institute for Enzyme Research and Department of Anthesiology, University of Wisconsin, Madison, Wisconsin 53706

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Abstract

Configurational changes of glutaraldehyde fixed heavy beef heart mitochondria are confirmed using the freeze fracture technique. Large amplitude swelling occurred after unfixed mitochondria were suspended in 30% glycerol. Fine structure of the outer and inner mitochondrial membranes is described using unfixed heavy beef heart mitochondria by the freeze fracture technique. The matrix side of the inner membrane appears to be covered with 90 Å particles while the opposite side (cytochrome *c* side) is also particulate covered by a high density of lower profile particles with a smooth underlying mosaic layer beneath. The outer surface of the outer membrane is smooth with particles embedded within the membrane. Possible structure of the membrane is discussed.

Introduction

Distinct mitochondrial configurational states have been observed by Hackenbrock [1] with isolated rat liver mitochondria, Green *et al.* [2, 3, 4] with isolated heavy beef heart mitochondria, and Harris *et al.* [5] and

* Present address: Department of Biophysics, Pennsylvania State University, University Park, Pennsylvania.

Williams *et al.* [6] with mitochondria *in situ* from a variety of tissues from different animal species. All of the above studies were done with glutaraldehyde fixed specimens using thin sections. From these and other studies, the configurational state of the mitochondrion seems to reflect the energy state as defined by Chance and Williams [7].

We wish to present evidence that identical configurational changes can be visualized with isolated heavy beef heart mitochondria using the freeze fracture technique with glutaraldehyde fixed specimens. We wish further to present evidence of the fine structure of the inner and outer membranes of unfixed heavy beef heart mitochondria using the freeze fracture technique of electron microscopy.

Materials and Methods

Heavy beef heart mitochondria were isolated by the method of Hatefi and Lester and placed in one of the four configurational states by the method of Green *et al.* [4]. Samples were fixed by adding equal volumes of a solution 2% in glutaraldehyde, 0.25 M in sucrose, and 50 mM in potassium cacodylate, pH 7.5, to the reaction mixture and a further incubation for 5 min at room temperature with vigorous stirring. The procedure for thin sections and freeze fracture of glutaraldehyde-fixed specimens were identical with those of Vail and Riley [9] with the exception that 30% glycerol was used as the cryoprotectant.

For freeze fracture of non-fixed specimens, mitochondria were washed three times in a solution 30% v/v in glycerol at 4° with 20 min incubation in each wash. The final suspension contained 50 mg protein per ml. Samples were placed in gold cups, frozen in Freon 22, stored no longer than 5 min in liquid N_2, and placed on the specimen table of a Balzer freeze etch apparatus (BA 360) precooled to −150°. All specimens were fractured at −100° at $2 \times 10\ 10^{-6}$ Torr and the specimens were immediately shadowed after the last fracture. Replicas were coated and washed by the method of Vail and Riley [9]. Replicas were mounted on bare 300 x 75 mesh copper grids and examined in a Philips EM-300 electron microscope operated at 60 kV. In all figures an arrow in a circle indicated the direction of the carbon-platinum shadow, and we have estimated a Pt deposit of 10 Å. Particle size determinations were corrected for Pt deposit.

Results

Distinct morphological configurational changes of heavy beef heart mitochondria as defined by Green *et al.* [4] are described. We have presented thin sections from the same sample for comparison with the freeze etch replicas. Figure 1 shows mitochondria in the non-energized aggregated state labelled to show surfaces in the freeze fracture replica

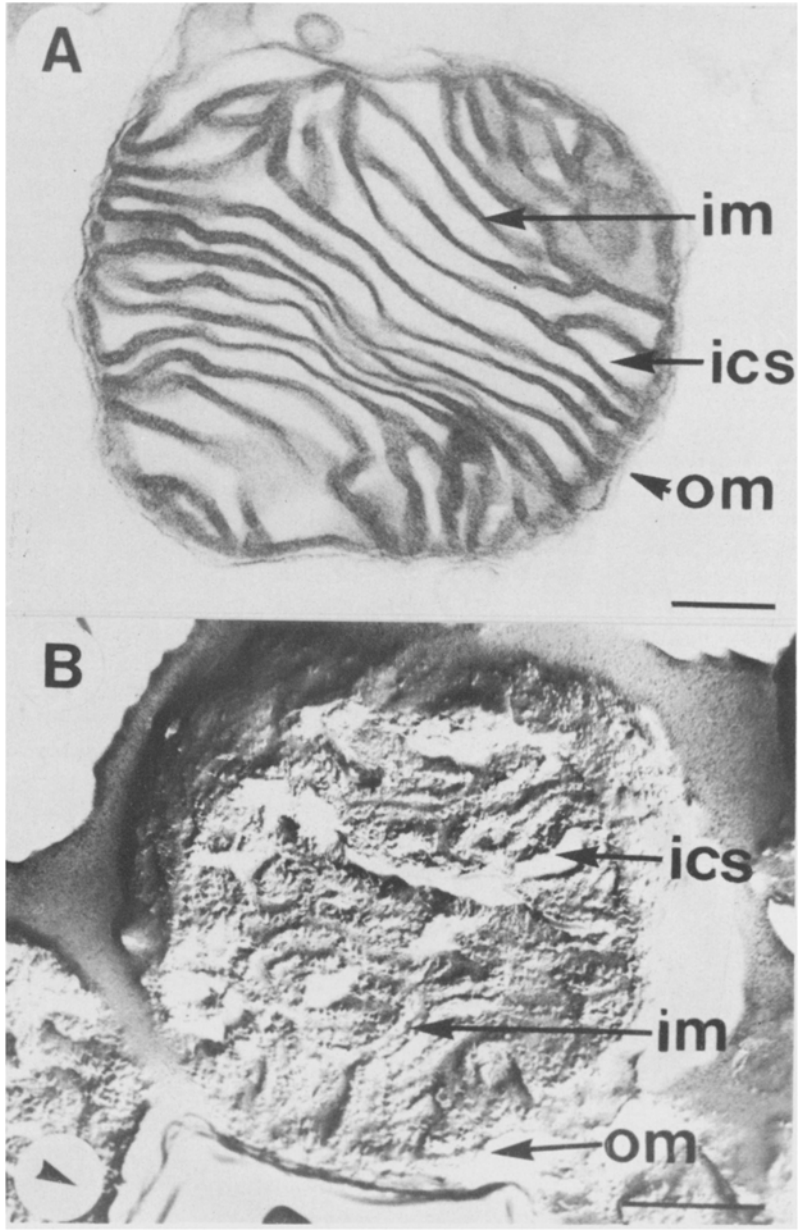

Figure 1. Heavy beef heart mitochondria fixed with glutaraldehyde in the non-energized aggregated state. IM—inner membrane, OM—outer membrane and ICS—intracristal space. Bar is 0.3 μ meters. (A) Thin section 57,500 x (UGDM 685-70). (B) Freeze fracture replica. Arrow indicated direction of shadow. Total magnification: 72,000 x (UGDM 929-P).

with their corresponding thin sections. It can be seen that beef heart mitochondria consist of two membrane systems, the outer membrane (OM) and the inner membrane (IM). Between the outer and inner membranes is the intracristal space (ICS) which is represented by an unstructured electron transparent region in thin sections. This space is also between the cristae (the invaginations of the inner membrane). The inner-most space of the mitochondrion is the matrix (M) which shows some grey electron density. It must be remembered, however, that each membrane has two surfaces. We have used the nomenclature of Racker [10] for the sides of the inner membrane. Thus, the outer membrane has a surface exposed to the outside environment (outer surfaces of the outer membrane). Likewise the inner membrane has two surfaces, that exposed to the intracristal space, cytochrome *c* side (the outer surface of the inner membrane) and the matrix surface, M-side (the inner surface of the inner membrane). The diagnostic characteristic of the non-energized aggregated state is the expanded intracristal space while the matrix space is contracted to a minimum. The inner membrane has sheet-like cristae which in some areas have been slightly twisted so that tangential sections along the same crista are obtained as well as cross sections.

When the mitochondrion becomes energized with a substrate such as succinate or malate and glutamate (Fig. 2) the matrix space swells slightly with a concomitant contraction of the intracristal space, the cristae tend to straighten to form parallel arrays of sheets of membranes. The electron density of the matrix becomes less.

When inorganic phosphate is added to a mitochondrial population already in the energized state, the cristae evaginate in many regions along the same crista to form tubes. The diagnostic characteristic of this state is the tubular form of the energized twisted state (Fig. 3). Both the freeze fracture and the thin section shows clearly the tubular cristae. It will be noted that through this transition the matrix space is further expanded.

If large amounts of uncoupler [9] or endotoxin [11] are added to mitochondria and an oxidizable substrate is added, there will be a further expansion or swelling of the matrix with the formation of the orthodox state (Fig. 5). If heart tissue is permitted to go anaerobic before fixation, the same configurational state can be visualized *in situ* [6].

We wondered what effect glycerol had on the gross morphology of the mitochondrion. A mitochondrial suspension at a protein concentration of 50 mg/ml was divided into two parts. One part was fixed by adding equal volumes of a solution 2% in glutaraldehyde, 0.25 M in sucrose and 50 mM in potassium cacodylate, pH 7.5 and incubated with rapid stirring for 5 min at room temperature. The sample was washed two times with the above solution without glutaraldehyde and placed in 30% glycerol at 4° for 30 min. The other half of the sample was incubated for 30 min at 4° in 30% glycerol and then fixed by adding the glutaraldehyde, sucrose,

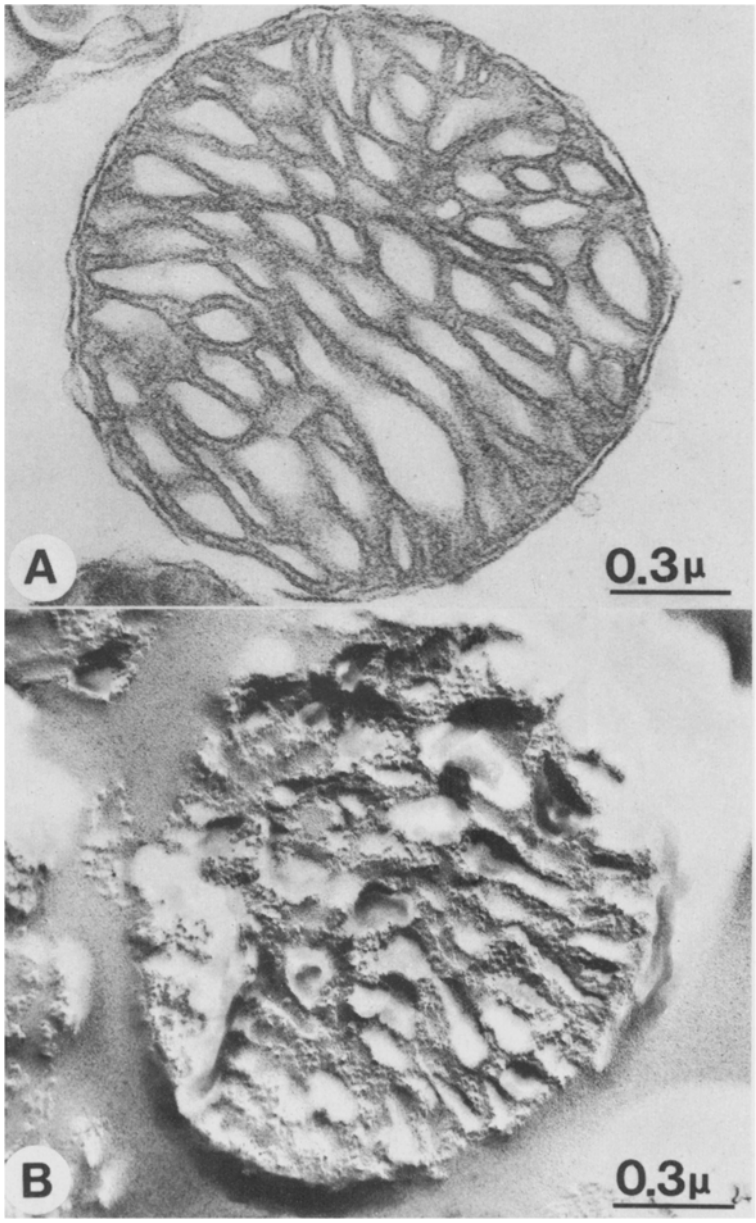

Figure 2. Heavy beef heart mitochondria fixed with glutaraldehyde in the energized aggregated state. Bar indicates 0.3 μ meters. (A) Thin section 73,600 x (UGDM 677-70). (B) Freeze fracture replica. Total magnification: 76,000 x (UGDM 176-P).

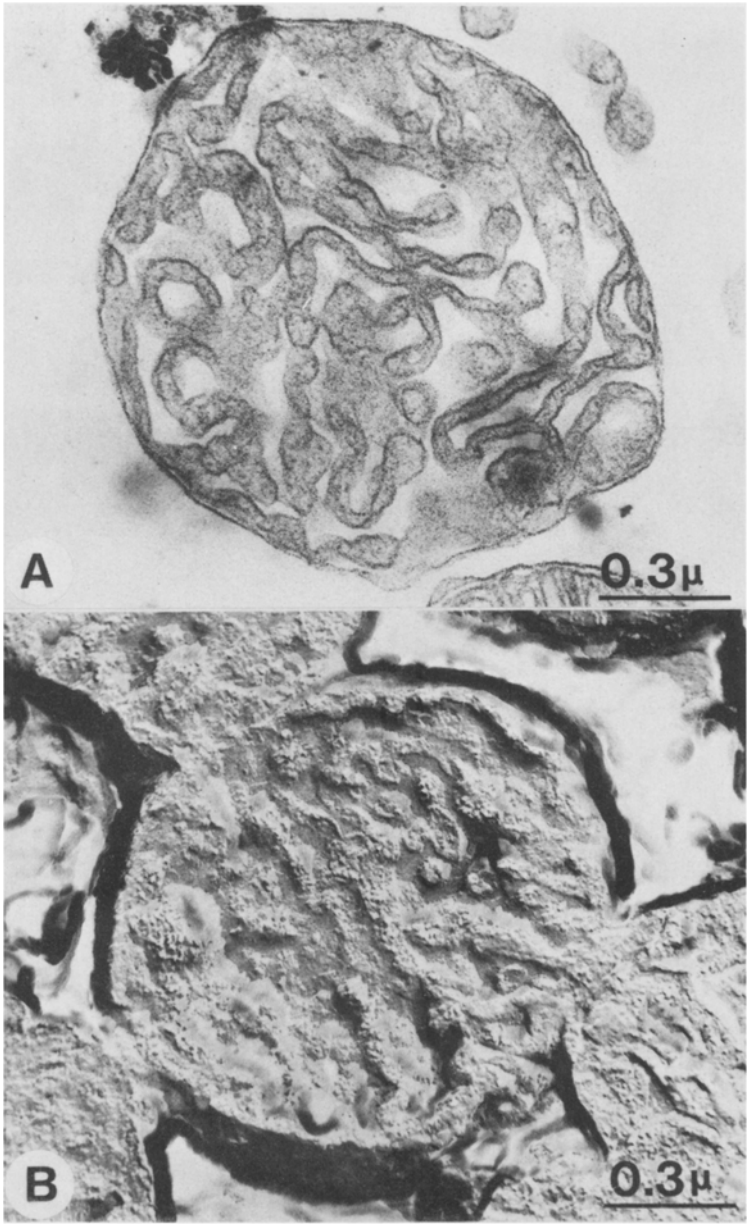

Figure 3. Heavy beef heart mitochondria fixed with gluataraldehyde in the energized-twisted aggregated state. Bar indicates 0.3 μ meters. (A) Thin section 80,000 x (UGDM 678-70). (B) Freeze fracture replica. Total magnification: 80,000 x (UGDM 259-P).

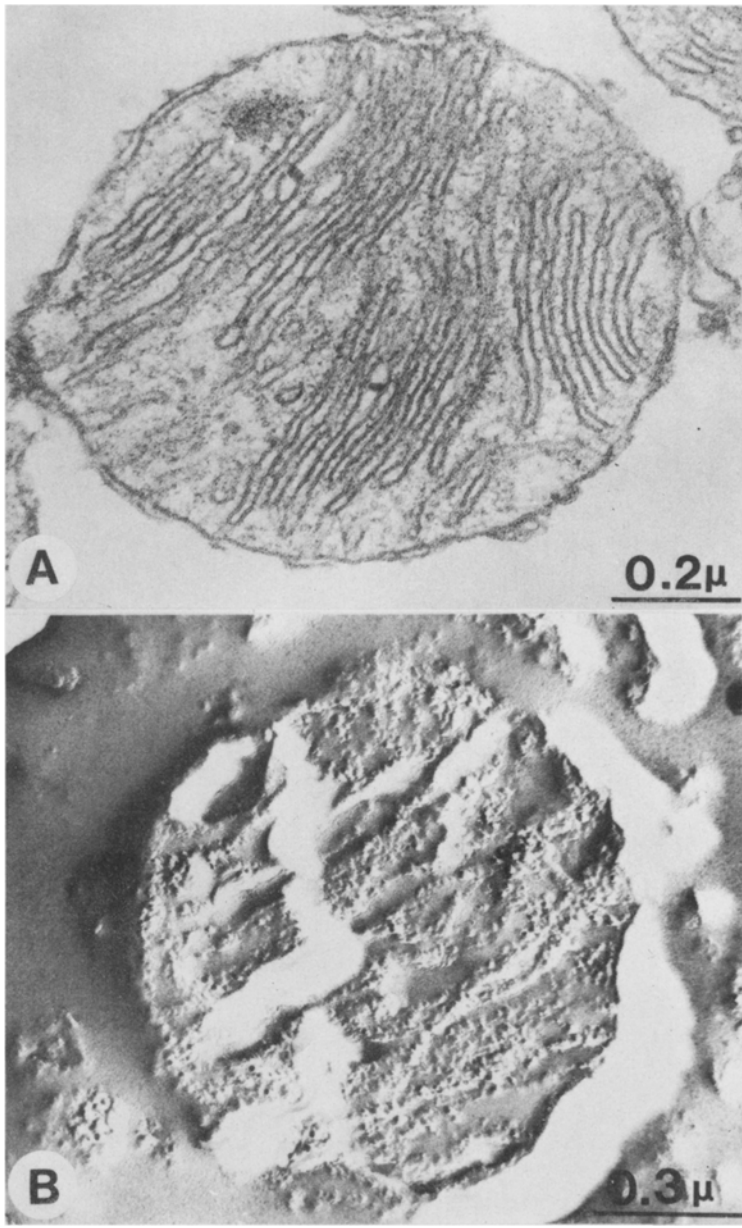

Figure 4. Heavy beef heart mitochondria fixed with glutaraldehyde in the non-energized orthodox state. Bar indicates 0.3 μ meters. (A) Thin section 80,000 x (UGDM 1476-P). (B) Freeze fracture replica. Total magnification: 94,000 x (UGDM 2439-70).

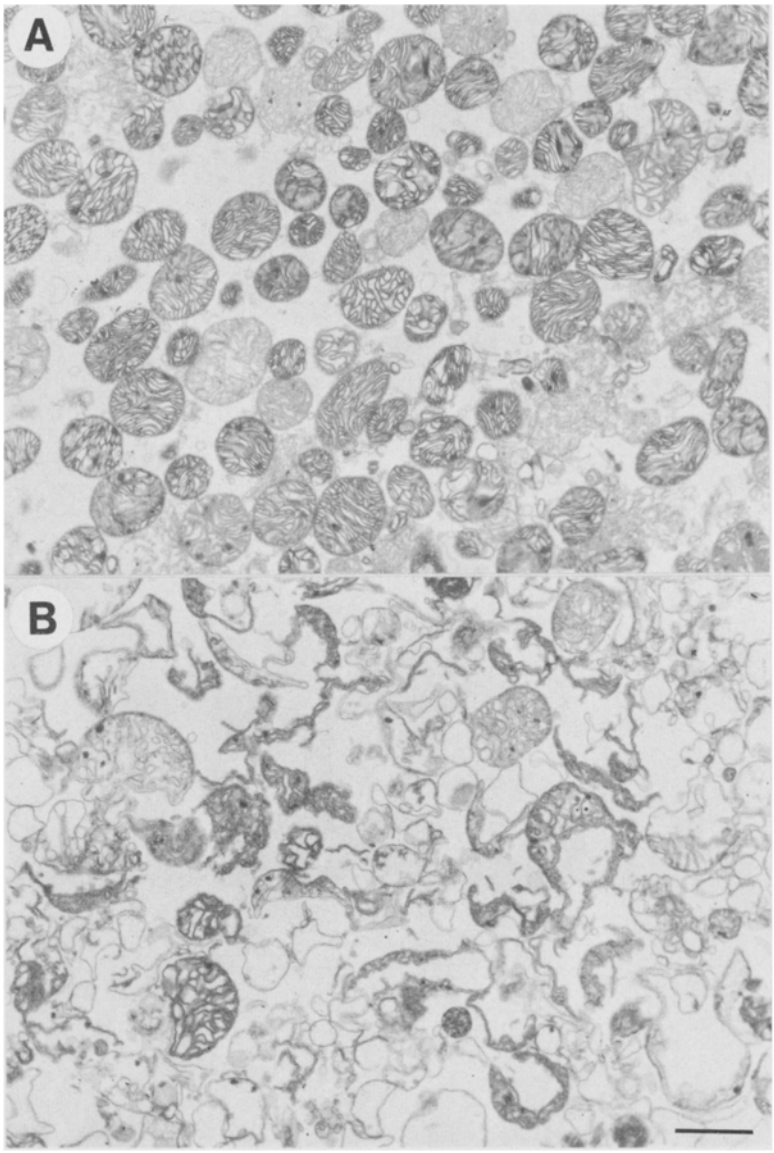

Figure 5. Heavy beef heart mitochondria in thin section. Total magnification: 15,600 x. Bar is 1 μM. (A) Glutaraldehyde fixed mitochondria and suspended in 30% glycerol at 4° for 30 min (UGDM 3707) (B) Unfixed mitochondria suspended in 30% glycerol at 4° for 30 min and then fixed with glutaraldehyde. Notice the large amplitude swelling (UGDM 3716).

cacodylate solution and incubated as above. Samples were then washed two times in sucrose cacodylate, stained, embedded and sectioned.

Figure 5 shows the results of this experiment. Figure 5A shows fixed mitochondria before the glycerol incubation. The mitochondrial population look aggregated and generally unswollen. However, after glycerol incubation the population of unfixed mitochondria (Fig. 5B) show large amplitude swelling. Most of the mitochondria have the outer membrane broken while only a few are intact but are swollen. It appears that even at 4°, 30% glycerol does not give sufficient osmotic support to suppress gross swelling which suggests complete permeability of the glycerol.

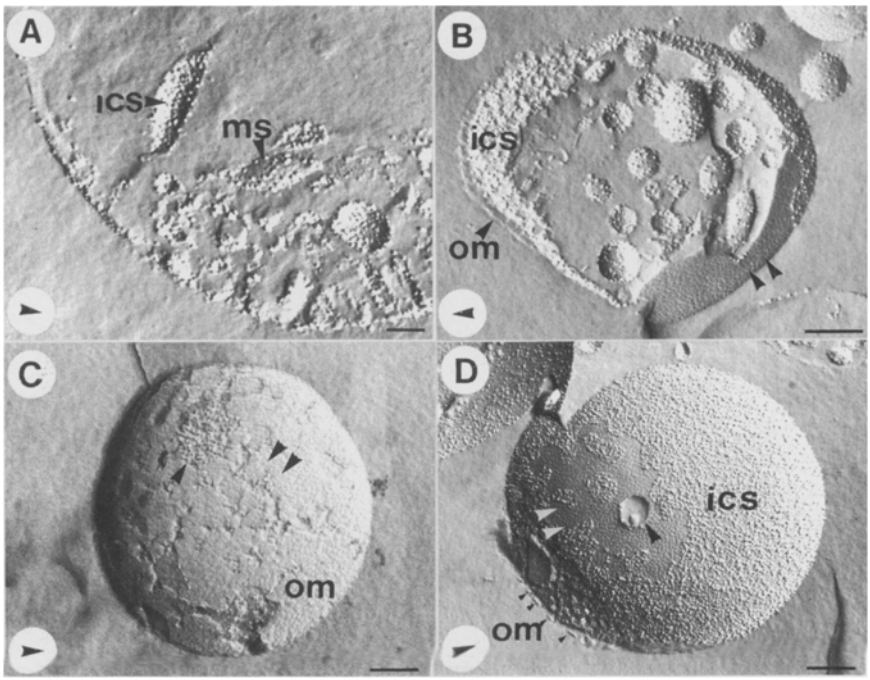

Figure 6. Freeze fracture of unfixed mitochondria suspended in 30% glycerol. (A) Intracristal surface (ICS) on cytochrome *c* side of the inner membrane, matrix surface (MS) of the inner membrane. Total magnification: 92,500. Bar is 1000 Å (UGDM 3356). (B) Tangential fracture of a mitochondrian showing outer membrane OM, intracristal surface (ICS) or cytochrome *c* side of the inner membrane with a smooth mosaic surface beneath (double arrows). Total magnification: 72,000 x. Bar 2000 Å (UGDM 3381). (C) Outer surface of the outer membrane showing particles embedded in the membrane (at arrows). Total magnification: 78,000 x. Bar is 1500 Å (UGDM 3410). (D) The outer surface of the intracristal surface (ICS) or cytochrome *c* side of the inner membrane covered with particles. Arrow shows what may be an invagination of a crista. Remnants of the outer membrane (OM) can be seen at small arrows. Total magnification 56,000 x. Bar is 2000 Å (UGDM 3383).

Figure 6 shows freeze fractures of unfixed heavy beef heart mitochondria. In Fig. 6A details of the inner membrane at a nearly median fracture can be seen as a concave fracture surface which we interpret to be the intracristal surface (ICS) or cytochrome *c* side of the membrane which has numerous low profile particles projecting from the membrane. These particles measure 70-90 Å in height (corrected for a Pt deposit of 10 Å). The convex surface (MS) or matrix surface has higher and larger particles (90-100 Å corrected for Pt).

At a tangential fracture in Fig. 6B the broken outer membrane (OM) is visible as a distinct line around one side of the swollen mitochondrion. The intracristal surface (ICS) is exposed by the fracture; however, another fracture surface (at double arrows) can be seen. This surface appears as a mosaic but relatively smooth surface which seems to be beneath the otherwise particulate intracristal surface.

Because of the high amplitude swelling induced by glycerol which causes breakage of the outer membrane, we were fortunate to have observed in Fig. 6C what we interpret as the outer membrane (OM) with fractures through it exposing the interior particulate region (at arrow). It is to be noted, however, that the fracture seems to expose the outer surface of the outer membrane which appears as a layer with small indentations as if a smooth layer was covering a particulate center core. This is opposed to the mosaic layer in Fig. 6B which is a smooth layer having depressions, as if particles were pulled from the smooth layer.

Figure 6D shows a swollen mitochondrion with at least two different fracture planes. The outer fracture surface appears to be the particulate intracristal surface (ICS) with the depressed mosaic surface beneath (white arrows). Small arrows show remnants of the outer membrane (OM) while single black arrow shows what could be a cristal invagination from the inner membrane.

Discussion

Using two distinctly different electron microscopic techniques from the same sample, we have presented evidence that heavy beef heart mitochondria undergo distinctive configurational states independent of the technique used. The state can be visualized by both thin sections [2, 3, 4] as well the freeze fracture technique [9].

Korman *et al.* [13] have postulated correlated membrane orientation with the change in state of heavy beef heart mitochondria using exhaustive analyses of thin section preparations. Our findings with the freeze fracture technique support their work. The cristae appear as sheet-like membranes in the non-energized aggregated (Fig. 1) and the energized aggregated state (Fig. 2). According to Korman *et al.* [13] analyses, the energized-twisted aggregated state appear as a series of tubules which straighten and align closely with adjacent tubules, the non-energized orthodox state (Fig. 4).

In order to preserve the configurational state in our preparations, it necessitated the use of the fixative generally used in electron microscopy, namely glutaraldehyde. However, it is doubtful whether glutaraldehyde has any pronounced effect other than "freezing" the configurational state for gross morphological examination. We have observed an aggregated state by exposing unfixed mitochondria to 0.5 M sucrose using the freeze fracture technique [17].

Glutaraldehyde is known to be an effective cross-linking reagent within proteins [14] and between adjacent proteins. This substance has been extensively used as an electron fixative [15]. With glutaraldehyde fixed specimens and the freeze fracture technique, we conclude that fractures rarely occur through the membrane and membrane surfaces are commonly seen. Glutaraldehyde treatment does produce a granularity in the replica while different fracture planes are present, namely around the membrane surface.

We have observed dense aggregates of particles with the freeze etch technique [16], if a suspension of ETP_H (a vesicular inner membrane preparation) is fixed with glutaraldehyde at concentrations greater than 1 mg protein per ml of solution. However, if the particle suspension is diluted to less than 0.5 mg protein per ml of solution, distinct isolated particles are present. We have taken these observations to further suggest that individual protein are exposed on the outside surface of ETP_H membrane (the M-side of the inner membrane) which would be cross linked with adjacent particles with glutaraldehyde if the particles are in close proximity with one another. However, even if heavy beef heart mitochondria are suspended at concentrations of 50 mg protein per ml of solution and fixed by adding small amounts of 25% glutaraldehyde–50 mM potassium cacodylate, pH 7.5, the fixed mitochondrial suspension can be diluted with solution so that isolated, individual mitochondria are visible using the freeze fracture technique.

Using unfixed mitochondria, pronounced swelling occurs with glycerol. Care should be taken to interpret freeze fractures of unfixed specimens, especially in light of gross morphological changes. Swollen mitochondria most probably have stresses placed on the membranes during swelling. Generally, with heart mitochondria, the outer membrane breaks but still adheres to a region of the mitochondrion. At this region of adherence, cristae can still be observed in the inner membrane. Most of the inner membrane is exposed to form a large vesicle [16].

The outer membrane and the inner mitochondrial membrane vary considerably in the ratio of phospholipid to protein. Parsons *et al.* [18] has shown that this ratio is 0.88 mg phospholipid to protein in the outer membrane as compared to 0.28 mg phospholipid to protein in the inner membrane. Thus fractures readily occur through the outer membrane in unfixed mitochondria, revealing a particulate core. However, we have observed few fractures through the inner membrane.

One possible explanation could be the high density of proteins in the membrane and probably relatively strong protein to protein bonds within the membrane. The hydrophobic bonds holding together the outer membrane would not exist at freeze etch temperatures (−100°), thus fractures through the membrane, while ionic bonds between proteins in the inner membrane might resist fractures.

The mosaic surface which is present beneath the particulate outer surface of the inner membrane could represent a phospholipid monolayer in which the particles and the other half of the bilayer have been removed by the fracture. However, this surface has yet to be fully determined.

Wrigglesworth *et al.* [18] has shown the structure of unfixed rabbit heart mitochondria, and we confirm their interpretation using heavy beef heart mitochondria, a smooth surface of the outer membrane covering a particulate surface of the C-side of the inner membrane. Their interpretation of rat liver mitochondrial inner membrane suggests that the ATPase (F_1) is normally within the membrane continuum, and the particle is exposed by the fracture through the membrane. This is not in agreement with our observations of the M-side of the inner membrane. Unless a protein conformational shape change is inferred, the spherical 90 Å ATPase molecule would not entirely fit into the membrane assuming 72 Å thickness from thin sections. We have observed that M-side of the inner membrane is covered with particles. The distribution of the 90 Å particles is lower than the distribution of particles on the C-side of the membrane. However, Ruska and Ruska [19] suggest that the outer surface of the inner membrane is not exposed in heart muscle mitochondria.

It seems premature to generalize as to the fracture planes either through or around membranes since only two membrane fracture planes have yet been determined [21, 22] both of which are red blood cell ghosts.

From our observations with membranes of heavy beef heart mitochondria using the freeze fracture technique, we have seen two distinct species of particles, those located on the surfaces of the inner membrane (extra membrane particles) and those particles located within the membrane (intermembrane particles). The latter is common to both the outer and inner membranes. It would be folly to suggest that all biological membranes possess both types of particles, but perhaps the common structure of the membranes is the intermembrane particulate structure.

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References

1. C. R. Hackenbrock, *J. Cell Biol.*, 37 (1968) 345.
2. J. T. Penniston, R. A. Harris, J. Asai and D. E. Green, *Proc. Nat'l. Acad. Sci., U.S.A.*, 59 (1968) 624.
3. R. A. Harris, J. T. Penniston, J. Asai and D. E. Green, *Proc. Nat'l. Acad. Sci., U.S.A.*, 59 (1968) 830.
4. D. E. Green, J. Asai, R. A. Harris and J. T. Penniston, *Arch. Biochem. Biophys.*, 125 (1968) 684.
5. R. A. Harris, C. H. Williams, M. Caldwell, D. E. Green and E. Valdivia. *Science*, 1965 No. 394 (1969) 700.
6. C. H. Williams, W. J. Vail, R. A. Harris, M. Caldwell, D. E. Green and E. Valdivia, *Bioenergetics*, 1 (1970) 147.
7. B. Chance and G. R. Williams, *Advances in Enzymol.*, 17 (1956) 65.
8. Y. Hatefi and R. L. Lester, *Biochim. Biophys. Acta*, 27 (1958) 83.
9. W. J. Vail and R. K. Riley, *Nature* 231 (1971) 525.
10. E. Racker, "The Two Faces of the Inner Mitochondrial Membrane' in *Essays in Biochemistry*, Campbell and Dickens (eds.), Academic Press, London (1970) 1.
11. R. A. Harris, D. L. Harris and D. E. Green, *Arch. Biochem. Biophys.*, 128 (1968) 219.
12. W. J. Vail, R. K. Riley and L. J. Collett (submitted for publication).
13. E. F. Korman, A. D. F. Addink, T. Wakabayashi and D. E. Green, *Bioenergetics*, 1 (1970) 9.
14. F. M. Richards and J. R. Knowles, *J. Mol. Biol.* 37 (1968) 231.
15. P. D. Sabatini, K. Bensch and R. J. Barrnett, *J. Cell Biol.*, 17 (1963) 19.
16. J. Asai, G. A. Blondin, W. J. Vail and D. E. Green, *Arch. Biochem. Biophys.*, 132 (1969) 524.
17. Unpublished observations.
18. D. E. Parsons, G. R. Williams, W. Thompson, D. Wilson and B. Chance, in: *Round Table Discussion on Mitochondrial Structure and Compartmentation*, E. Quagliariello, S. Papa, E. C. Slater and J. M. Tager (eds.), Adriatica Editrice, Bari (1967) 29.
19. J. M. Wrigglesworth, L. Packer and D. Branton, *Biochim. Biophys. Acta*, 205 (1970) 125.
20. E. Ruska and H. Ruska, *Z. Zellforsch.*, 97 (1969) 298.
21. P. P. da Silva and D. Branton, *J. Cell Biol.*, 45 (1970) 598.
22. T. W. Tillack and V. T. Marchesi, *J. Cell Biol.*, 45 (1970) 649.