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CHANGES IN FREEZE-FRACTURED MITOCHONDRIAL MEMBRANES CORRELATED TO THEIR ENERGETIC STATE

DYNAMIC INTERACTIONS OF THE BOUNDARY MEMBRANES

GERHARD KNOLL and DIETER BRDICZKA *

Fakultät für Biologie der Universität Konstanz, D-7750 Konstanz (F.R.G.)

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Structural changes of mitochondria in correlation to their energetic state have been observed as matrix expansion and condensation. In this communication we describe a morphological correlation in freeze-fractured mitochondrial membranes which is also dependent on the metabolic state of the organelle: the frequency by which the fracture plane following the inner or outer boundary membrane deviates by jumping from one membrane to the other is higher in phosphorylating mitochondria when compared to freshly isolated or energized mitochondria. These deflections of the fracture plane occur mostly in minimal, short steps showing close apposition of the two boundary membranes. We therefore conclude that the observed change in morphological appearance is produced by a change in interactions between the inner and the outer membranes correlated to the different functional states of the inner membrane.

Introduction

Inner membrane-matrix condensation and expansion in isolated mitochondria correlated to their energetic states have been described and widely characterized in thin sections by Hackenbrock [1,2]. Several investigations have attempted to extend these morphological studies to the membranes by applying freeze-fracture techniques, as this method, in particular, yields much information about the events occurring in the plane of the membrane. However, to our knowledge, no investigation of the fracture faces of mitochondrial membranes in different energetic states has been made which avoids the use of glycerol and/or glutaraldehyde fixation. Lang and Bronk [3] have analysed the kinetics of the orthodox to condensed transition in unfixed and unglycerinated liver mitochondria by applying the spray-freeze method. However, their results were based on the examination of cross-fractured mitochondria and no effort was made to analyse the membrane faces in the different mitochondrial states. Glycerol and glutaraldehyde have been shown to cause severe artefacts, especially in the field of delicate membrane-membrane interactions [4,5].

Recently, much progress has been made in the reduction of the time necessary for freezing of a specimen. By the new very rapid freezing techniques, redistribution of ions, lipids and proteins and the growth of ice crystals are reduced, thus allowing cryofixation without glutaraldehyde and cryoprotectives [6].

* To whom correspondence should be addressed.
Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

In this investigations we have applied pure physical fixation by rapid freezing to isolated mitochondria in different energetic states. We have observed that the mitochondrial morphology is different as compared to thin sections and in addition we have realized a new morphological correlate which has not been previously observed because of its being almost absent when cryoprotectives such as glycerol are used. This morphological structure is a change in the course of the fracture plane in the two boundary membranes. We have analysed the frequency by which the fracture plane deflects and jumps to another layer and observed that it correlates very well with the activity of oxidative phosphorylation [7].

Experimental procedures

Materials

Rats, strain Chbb:THOM fed with a standard diet were used for all experiments.

ADP was obtained from Boehringer-Mannheim; all other reagents were p.a. grade and were purchased from Merck, Darmstadt, F.R.G.

Methods

Liver mitochondria were isolated from rats in a medium comprising 0.25 M sucrose/10 mM Hepes (pH 7.4)/0.1 mM phenylmethylsulfonylfluoride/0.1 mM EGTA. Mitochondria suspended in this medium were used as control (state 1) mitochondria. Hypo- or hypertonic conditions were established by suspending the mitochondria in the same medium as above containing 0.125 M or 0.35 M sucrose, respectively.

During the mitochondrial preparation the temperature was 4°C whereas the subsequent procedures were performed at room temperature.

The orthodox and condensed states of the mitochondria were adjusted essentially as described by Hackenbrock [1] and were monitored using a Clark oxygen electrode. 1 ml aliquots of the monitored mitochondrial suspensions were aerated by stirring and subsequently centrifugated for 10 s in a Beckman Minifuge at $8700 \times g$.

Cryofixation. The pellets were subjected immediately to rapid cryofixation as described recently [8,9] in order to avoid anaerobiosis. Cryofixation lasts 10 s. Routinely, four samples were prepared. Therefore, the maximal time including centrifugation which was needed for fixation of the last sample was 60 s. Higher centrifugal force was applied to some samples by prolongation of the centrifugation time to 1 min.

Freeze-fracture. The sandwiches were broken in a Balzers 360 M freeze-etch device equipped with an adapted Balzers double replica table at -120°C and $2 \cdot 10^{-7}$ Torr followed by Pt/C and C shadowing.

Electron microscopy. A Siemens 101 instrument operated at 80 kV was used for electron microscopy. The morphological evaluations were performed using a Kontron MOP Am2 picture analysing system connected to a Hewlett-Packard 9825 calculator.

The nomenclature of the exposed membranes follows the proposals of Branton et al. [10].

Results

Identification of the fracture planes

Freeze-fractured isolated mitochondria and mitochondria in intact cells exhibit a morphological structure different from that of all other cell organelles. This structure is characterized by the irregular course of the fracture plane jumping back and forth between two different layers. In order to realize the structure and function which is responsible for the fracture plane deflections, an understanding of how the faces of the two boundary membranes are exposed by fracture and by etching is required. One way to identify the fracture faces is to analyse organelles which are in part cross-fractured. For example, in the three convex fractures of mitochondria in state 3 shown in Fig. 1a–c, the fracture face of the smooth membrane covering the second membrane continues in the cross-fractured outer membrane. Two different membrane faces are also exposed in the two concave fractures of state 3 mitochondria shown in Fig. 1d–e. As the cross-fractured edge of the deeper membrane continues with the respective membrane face, it seems plausible that the upper face belongs to a second attached membrane. From the convex fractures in Fig. 1a–c the conclusion can be drawn that the smooth membrane covering the inner membrane represents outer membrane. Therefore, in the concave fractures the attached

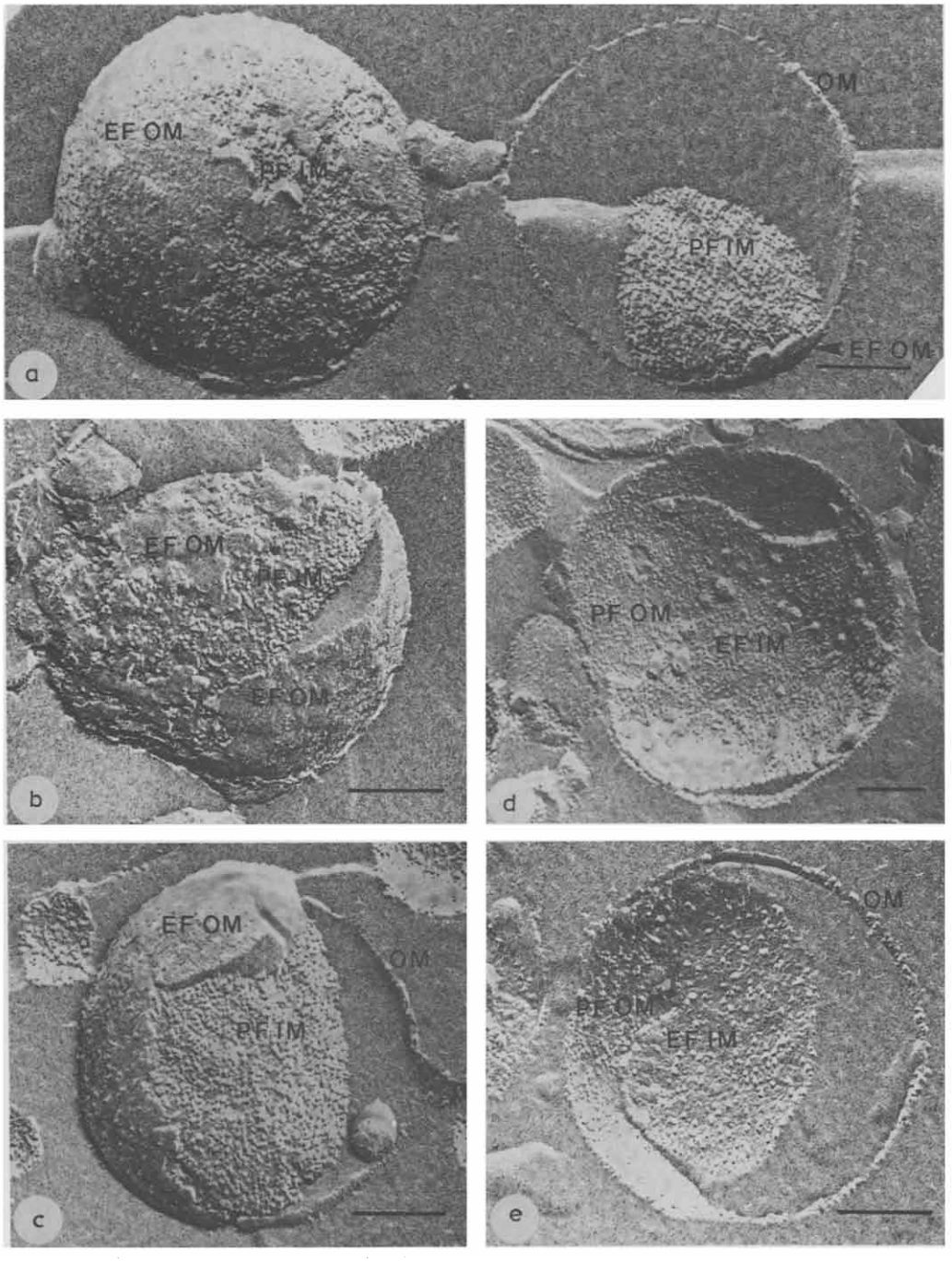

Fig. 1. Convex and concave fractures of isolated phosphorylating mitochondria (state 3). In the four convex fractures, a–c, the fracture face of the smooth membrane covering the inner membrane continues in the cross-fractured outer membrane. In the two concave fractures, d, e, the deeper membrane represents the outer membrane. Therefore, the second attached membrane is suggested to be the inner membrane. By comparing the concave and convex fractures, it seems evident that the exhibited planes represent the complementary halves of the fractured membranes. Bar = 0.2 μm. The different layers are designated according to the nomenclature in Ref. 10. OM = outer membrane, IM = inner membrane.

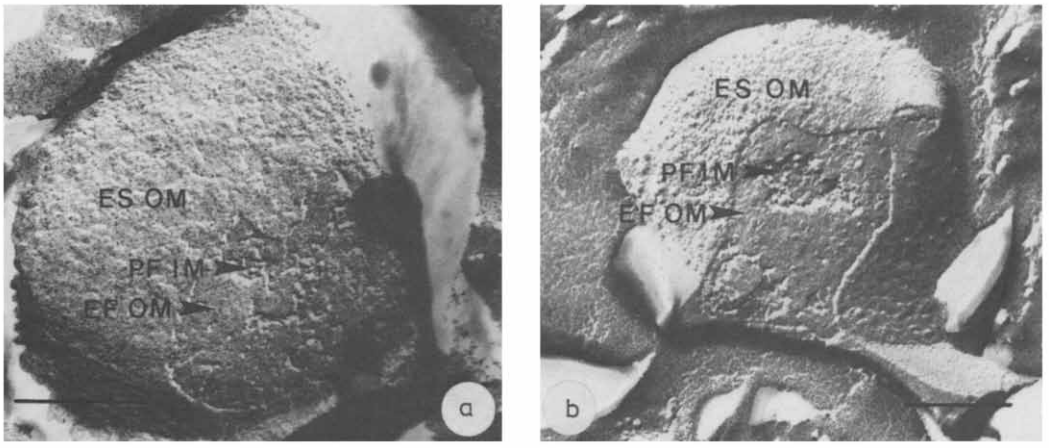

Fig. 2. Etching of freeze-fractured mitochondria in state 1. (a) Mitochondria suspended in distilled water. (b) Mitochondria partially suspended in the ice phase due to phase separation in the isolation medium during slow freezing (conventional, in Freon 22). In both pictures, a third new plane is exposed by etching which represents the true surface of the outer membrane. Bar = 0.2 μm.

second membrane should be the inner membrane.

By comparing the convex and concave fractures in Fig. 1, it seems evident that the exhibited planes represent the complementary halves of the fractured membranes. Considering this complementary, another important point can be raised, which is that the planes of the membranes exposed in the convex and concave fractures should then represent the inner faces of the outer or inner membrane. However, as it is still under discussion whether or not a fracture may also deflect from the surface to the interior of a membrane [11], we dealt with this question by etching a convex mitochondrial freeze-fracture. The true surface of the outer membrane should be exposed if the assumption holds that the fracture runs in the core of the membrane.

It is impossible to sublime highly concentrated solutions, as they are necessary to keep mitochondria under isotonic conditions. Therefore, we suspended mitochondria in destillied water, conditions under which most of them are disrupted – as the observation of parallel thin sections showed. In spite of this there remained some mitochondria with closely apposed membranes. Fig. 2a shows a mitochondrion exhibiting the habitual two faces together with a third new plane after deep etching which must represent the true surface of the outer membrane. Additional support for our interpretation is displayed by etched slow-frozen mitochondria of state 1: due to phase seperation during freezing, there are parts of mitochondria suspended in the ice phase, and here after etching a new plane is also exposed (Fig. 2b). In contrast to the mitochondrial freeze-etch pictures, isolated outer membrane in sucrose medium reveals one fractured and one etched face (not shown). Consistent with this layer identification is the estimation of particle density with regard to the different membrane halves. The relations correlate very well with the relative particle densities of the four faces of the two membranes established from various authors [12,13].

Structural changes correlated to metabolic states

From the identification of the different layers, it seemed evident that in mitochondria of state 3 (Fig. 1) and state 1 (Fig. 2), as well as in other metabolic states we have analysed (not shown), the fracture plane changes between the interior of both boundary membranes. Therefore, the uppermost layer in convex fractures is the exoplasmic face of the outer membrane, whereas in concave fractures it represents the exoplasmic face of the inner membrane. We observed that the frequency of deflections of the fracture plane changes in correlation with the metabolic state. There are more deflections of the fracture plane in phosphorylating mitochondria than in non-phosphory-

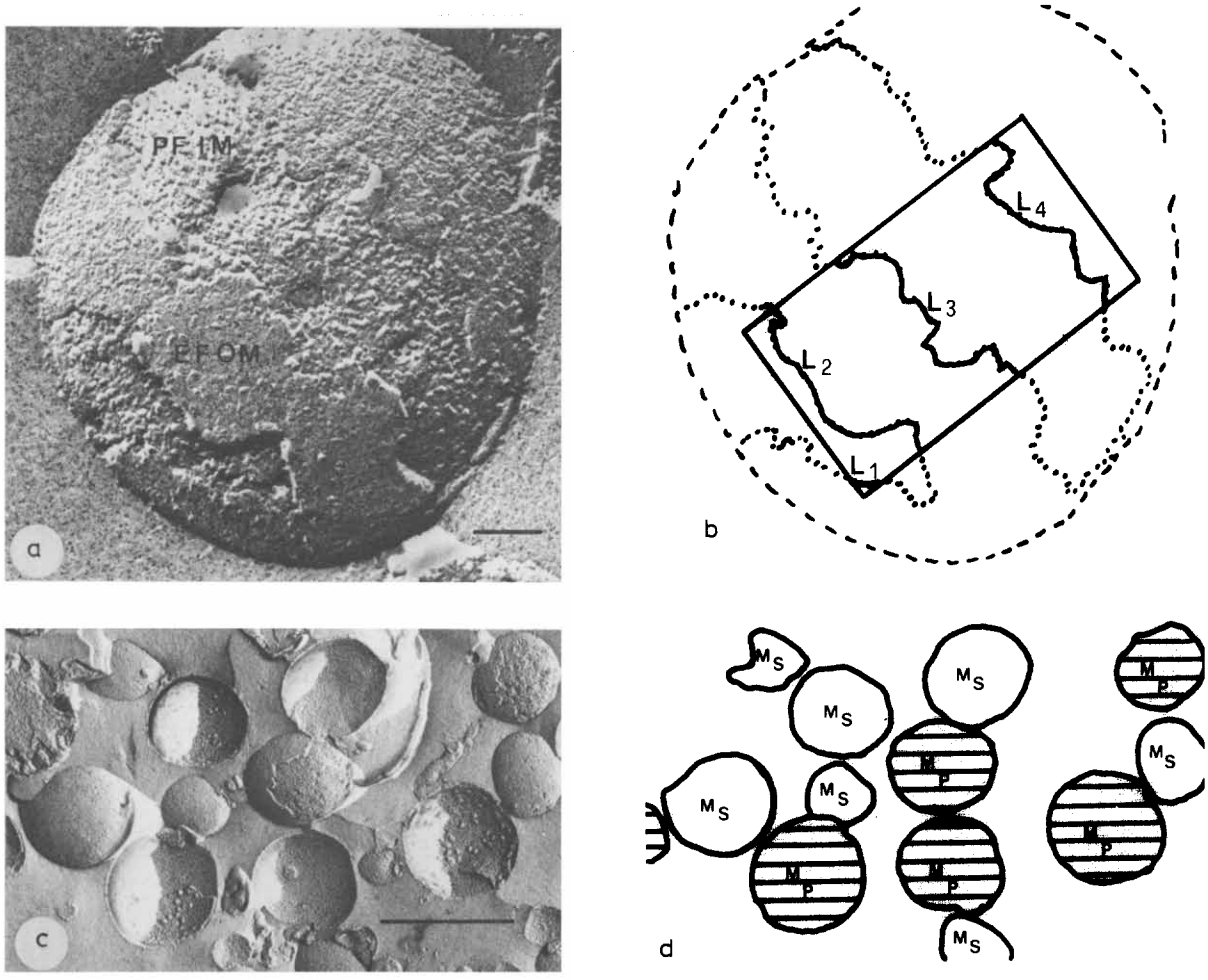

Fig. 3. (a) Freeze-fracture of a mitochondrium (state 1). Bar = 0.1 μm. (b) Scheme of the evaluation procedure. (c) Survey of freeze-fractured mitochondria (state 1). (d) Scheme of the evaluation procedure. Isolated mitochondria were adjusted to the respective energetic state, frozen and freeze-fractured as described above. From each preparation 10–20 detailed micrographs were taken showing mitochondria with fracture plane deviations and surveys with approximately 100 fractured mitochondrial membranes. As a means of quantifying the difference in fracture plane deviations, we measured the length of the edge where the fracture plane deflects (solid line in b) as related to the corresponding area (shown in b). The obtained values were a certain length (L) per area ($\mu m/\mu m^2$). The length of the edges was determined in an area where the platinum carbon was evaporated at an angle of approx. 45° to the plane of the specimen. In order to perform an analyisis which takes into consideration the different amount of smooth membrane fractures in the respective mitochondrial fractions, we determined on survey pictures the total area of smooth membrane fractures (M_s) and patchy membrane fractures (M_p) as shown in c and d. The single values, L, were then weighted by the factor $M_p/(M_p + M_s)$ for the particular preparation.

lating ones. As a means of quantifying the difference in fracture plane deviations, we measured the length of the edge where the fracture plane deflects as related to the corresponding area. As shown, for example in Fig. 3a and b, we measured in convex fractures the edge of the exoplasmic face of the outer membrane and in concave fractures (not shown) that of the exoplasmic face of the inner membrane. As shown in Fig. 3b, the quantification was made in areas where the curvature was low in order to avoid large distortions of the measured edge lines.

TABLE I

LENGTH OF THE FRACTURE-PLANE EDGE IN FREEZE-FRACTURED MITOCHONDRIA UNDER DIFFERENT CONDITIONS.

The evaluation was performed as described in Fig. 3, M_p represents the relative area of mitochondrial fracture faces exhibiting the described patches, L is the mean value of the individual length measurements of the fracture plane deviations and L_p is the weighted value with regard to the whole mitochondrial population. The different conditions are: (a) state 1 (freshly isolated mitochondria); (b) state 4 (energized by succinate); (c) state 3 (phosphorylating in the presence of succinate and ADP); (d) uncoupled (by the addition of 50 μM 2,4-dinitrophenol); (e) osmotically shrunken (in the presence of 350 mM sucrose); (f) osmotically swollen (in the presence of 125 mM sucrose); (g) pelleted by prolonged centrifugation; (h) incubation with 30% glycerol. Mitochondria e–h are in state 1. Number of different experiments: a–d, $n = 3$, e–h, $n = 1$. The significance of the difference between the values obtained under the described conditions – tested by the Kruskal-Wallis rank sums test as described in Ref. 27 – could be proved with $P < 0.01$. By Dunn's multiple comparisons based on the Kruskal-Wallis rank sums, the groups c, d, h could be shown to differ significantly from the control with $P < 0.01$. No significant difference of b, e, f, g compared to the control could be found, even at the level $P = 0.1$.

	a	b	c	d	e	f	g	h
M_p (%)	58	58	82	17	46	65	44	11
L (μm^{-1})	5.1 ± 2.7	5.9 ± 2.4	14.2 ± 7.0	2.8 ± 1.6	4.2 ± 1.5	5.2 ± 1.4	4.9 ± 1.8	3.3 ± 1.4
L_p (μm^{-1})	3.0	3.1	11.7	0.5	1.9	3.4	2.1	0.4

We additionally included in our measurements mitochondria without fracture plane deflections because, depending on the metabolic state, the proportion of mitochondria with patchy faces to those exhibiting smooth faces varied significantly. This shows that the morphological criterion of fracture plane deflections undergoes even more changes in correlation to the different metabolic states than could be determined on selected mitochondria. The relation between smooth and patchy faces was determined on survey pictures as shown in Fig. 3c and d and was included in the calculation as described in the legend to Fig. 3. The results given in Table I show that the length ($\mu m/\mu m^2$) of the fracture edge in condensed (state 3) mitochondria was 4 times that of state 4 and control (state 1) mitochondria.

In thin sections as described by Hackenbrock [1,2], phosphorylating as well as freshly isolated mitochondria have a condensed inner membrane-matrix compartment which leads to a separation of the two boundary membranes with only a few

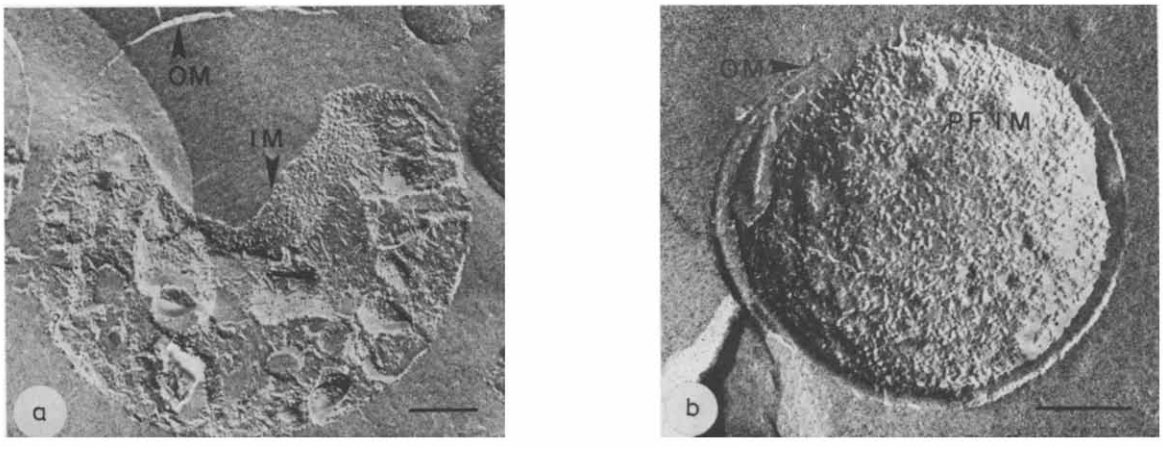

Fig. 4. Freeze-fracture of mitochondria in different metabolic states. (a) state 3 in the presence of succinate and ADP; (b) uncoupled by 50 μM 2,4-dinitrophenol. Mitochondria in the metabolic state 3 show a condensed inner membrane-matrix compartment, but, in spite of that, large parts of the two boundary membranes remain in close contact. Uncoupling leads to a complete separation of the boundary membranes. Bar = 0.2 μm.

membrane contacts remaining. This seems to be in contrast to the finding of an increase in fracture plane deflections in phosphorylating mitochondria. However, the appearance of reduced membrane contacts in thin sections seems to be an artefact produced by the chemical fixation because, as Fig. 4a shows, it is not observed in mitochondria fixed by rapid freezing. In the latter mitochondria, the inner membrane-matrix compartment is also contracted, as was determined quantitatively by Lang and Bronk [3], but in spite of this, large parts of the two boundary membranes remain closely attached. However, in response to condensation at the opposite site, the two membranes become seperated. If the mitochondrial membranes were cleaved in the latter region, no jumping of the fracture plane would be expected. A low number of smooth fracture faces is indeed observed in the phosphorylating mitochondrial sample.

Another morphological discrepancy with the thin sections is observed by studying the action of 2,4-dinitrophenol on freeze-fractured mitochondria. As documented in Table I, the frequency of fracture plane deflections is very low and comparable to that in glycerinated mitochondria. The reason for this is the absence of membrane contacts. As is shown in Fig. 4b, both boundary membranes are completely separated in most mitochondria. This is not observed in orthodox freeze-fractured mitochondria, whereas in thin sections, orthodox and 2,4-dinitrophenol uncoupled mitochondria appear to have the same morphology.

Influence of osmotic pressure and centrifugal force on the course of the fracture plane

From the work of Hackenbrock and Packer [1,2,14], it is evident that osmotic forces can change the morphology of the mitochondria in a manner comparable to conformational transformations in response to metabolic changes. On the other hand, it has been reported that centrifugal pressure influences the distribution of particles in the mitochondrial membrane [15]. It was therefore of interest to determine the influence of hypo- and hypertonicity and high centrifugal force upon the behaviour of fracture-plane deflections. The results of these experiments are documented in Table I. The different samples were analysed in the same manner as the mitochondria in different energetic states. Osmotically shrunken mitochondria as well as those subjected to high centrifugal force have a slightly reduced frequency of fracture-plane deflections, whereas osmotically swollen mitochondria are comparable to the controls. None of the three preparations, however, differs significantly from the control and state 4 mitochondria.

Discussion

In intact liver cells fixed by rapid freezing, mitochondria, unlike other cell organelles, show an irregular freeze-fracture face which is characterized by a frequent change of the fracture plane between different layers (unpublished results). This morphological appearance is preserved during isolation of the mitochondria, but is suppressed by glycerination.

By analysis of mitochondria in which the two boundary membranes are exposed, partially as fractured planes and partially as cross-fractures, two things became evident. First, that the fracture course changes between layers of both boundary membranes and, second, that probably no membrane surface is exposed. The latter conclusion was drawn from the fact that convex and concave fracture faces represent complementary halves of the fractured organelle. Thus, freeze-cleavage should always expose the inner face of the cleaved membrane halves. This assumption was additionally supported by visualising the true surface of the outer membrane upon etching.

A diagram representation is given in Fig. 5, which summarizes how we suggest the freeze-cleaved half membranes arise from the outer and inner membranes, respectively. Sjöstrand and Cassell [11], working with heart mitochondria, have suggested that the fracture planes represent the inner and outer surfaces of the same membrane. The patches covering the inner membrane were interpreted by them as representing lipid material belonging to the inner membrane, since they seemed to be located almost in the same plane. This is not seen in the fracture faces presented in this investigation. The differences may be caused by the different experimental conditions. The authors used glycerinated heart tissue which had been previously fixed by perfusion with 1%

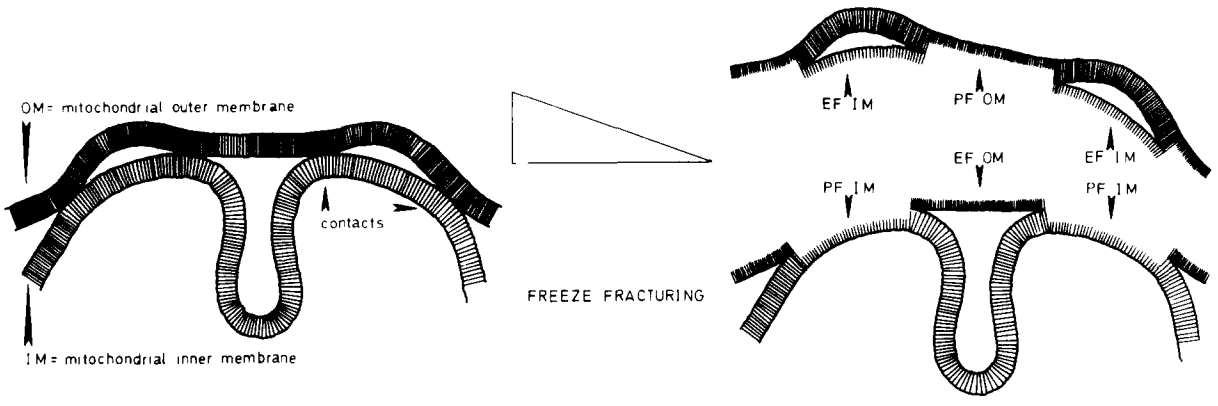

Fig. 5. Diagrammatic representation of how the freeze-cleaved half-membranes arise from the outer and inner membranes, respectively. The different layers are designated according to the nomenclature in Ref. 10.

glutaraldehyde. In spite of these results, the interpretation that the fracture plane jumps between the interior of both membranes has become widely accepted [12,16–18]. The implication of these results is that the fracture plane upon deflection has to cross the outer mitochondrial compartment. The course of a fracture like this can be imagined by assuming close contacts between the two boundary membranes.

Such contacts have been described by Hackenbrock in the case of thin-sectioned mitochondria [19]. Nothing is known about the nature of the contacts. Considering that the fracture plane follows hydrophobic regions, the contacts would represent zones in which the membranes are fused or connected by hydrophobic materials. An interpretation like that has been presented by Van Venetië and Verkleij [20], who proposed a semifusion model for the contact sites in which non-bilayer lipids are involved.

From the results presented above, it can be concluded that close proximity of the membranes alone is not effective in producing a high frequency of fracture-plane deflections, because by analysing mitochondria with closely apposed membranes (i.e., orthodox, state 4, and hypotonically swollen mitochondria) we observed no significant increase in fracture plane deflection frequency when compared to the controls. Therefore, besides close apposition between both membranes, an increase of fracture-plane deflections is produced by a second event which is presumably propagated by conformational changes of the inner membrane during oxidative phosphorylation. Otherwise, one cannot understand the significant increase of fracture-plane deflections which occurs exclusively in phosphorylating mitochondria. In view of these results, we suggest that the morphological criterion of fracture-plane deflection yields information about a structural organization and function which is restricted mainly to contacts between the boundary membranes and their dymamic changes correlated to the mitochondrial metabolism.

The criterion is therefore not strictly correlated to the condensed-orthodox transformations described. However, it has recently been shown [20] that the patch pattern used by the jumping of the fracture plane can be induced upon incubation of the mitochondria with Ca^{2+}, Mg^{2+}, Mn^{2+} and by incubation at 37°C. However, the metabolically induced difference in frequency of fracture-plane deflection seems to be independent of the presence of Mg^{2+} and inorganic phosphate, since these ions were present in the respective experiments.

Phosphorylating, as well as freshly isolated, mitochondria have a condensed inner membrane-matrix compartment which, according to Hackenbrock's work on thin sections, leads to a separation of the two boundary membranes with only a few membrane contacts remaining. This appearance is in contrast to the morphology of mitochondria fixed by rapid freezing where large parts of the inner and outer membrane remain in close contact, although as in thin sections the inner membrane-matrix compartment is contracted, On the other hand, complete separation of the

boundary membranes in uncoupled and glycerinated mitochondria is visualized exclusively by applying pure physical fixation and is not seen in conventional thin-sectioned material.

Although it is difficult to infer three-dimensional behaviour of structures from two-dimensional cross-fractured samples, it can serve to explain the increase in fracture plane jumps in phosphorylating mitochondria and the decrease in glycerinated and uncoupled organelles. In general, the differences between glutaraldehyde and pure physical fixation refer mainly to the interactions between the two boundary membranes, whereas the matrix expansion and condensation is the same.

The results presented here raise the question as to whether or not the contacts between the two boundary membranes play a regulatory role in vivo, for example in influencing activity of enzymes in the outer membrane. It has indeed been observed that the activity of monoamine oxidase in the outer membrane is affected by the respiratory state of the mitochondria (state 4, increased activity) [21].

Another observation which suggests a possible function of the contacts is that hexokinase upon binding to the mitochondrial surface seems to create a microcompartment facilitating a direct exchange of ADP and ATP between the enzyme and the compartment of oxidative phosphorylation [22]. The molecular basis of these observations has become clearer since we have provided evidence that hexokinase binds directly to the pore protein which renders the outer membrane permeable to ADP [23,24]. These results may serve to suggest a function of the contacts, that is, to connect the pore protein in the outer membrane directly to the ATP/ADP translocator in the inner membrane [25,26].

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References

1 Hackenbrock, C.R. (1966) J. Cell Biol. 30, 269–297
2 Hackenbrock, C.R. (1968) J. Cell Biol. 37, 345–369
3 Lang, R.D.A. and Bronk, J.R. (1978) J. Cell Biol. 77, 134–147
4 Chandler, D.E. and Heuser, I. (1979) J. Cell Biol. 83, 91–108
5 Rash, J.E. and Hudson, C.S. (eds.) (1979) Freeze fracture: Methods, Artefacts and Interpretations, Raven Press, New York
6 Plattner, H. and Bachman, L. (1982) Int. Rev. Cytol. 79, 237–304
7 Knoll, G. and Brdiczka, D. (1980) Eur. J. Cell Biol. 22, 281
8 Pscheid, P., Schudt, C. and Plattner, H. (1981) J. Microsc. 121, 149–167
9 Knoll, G., Oebel, G. and Plattner, H. (1982) Protoplasma 111, 161–176
10 Branton, D.S., Bullivant, S., Gilula, N.B., Karnovsky, M.J., Moor, H., Muehlethaler, K., Northcote, D.H., Packer, L., Satir, P., Speth, V., Staehelin, L.A., Steere, R.L. and Weinstein, R.S. (1979) Science 190, 54–56
11 Sjöstrand, F.A. and Cassell, R.Z. (1978) J. Ultrastruct. Res. 63, 111–137 and 138–154
12 Packer, L. (1973) in Mechanisms in Bioenergetics, (Azzone, G.F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds.), p. 77, Academic Press, New York
13 Losa, G.A., Weibel, E.R. and Bolender, R.P. (1978) J. Cell Biol. 78, 289–308
14 Packer, L. (1960) J. Biol. Chem. 235, 242–249
15 Wattiaux-DeConinck, S., Dubois, F. and Wattiaux, R. (1977) Biochim. Biophys. Acta 471, 421–435
16 Wrigglesworth, J.M., Packer, L. and Branton, D. (1970) Biochim. Biophys. Acta 205, 125–135
17 Melnick, R.L. and Packer, L. (1971) Biochim. Biophys. Acta 253, 503–508
18 Tewari, J.P., Tu, J.C. and Malhotra, S.K. (1972) Cytobios 5, 261–273
19 Hackenbrock, C.R. (1968) Proc. Natl. Acad. Sci. U.S.A. 61, 598–605
20 Van Venetië, R. and Verkleij, A.J. (1982) Biochim. Biophys. Acta 692, 379–405
21 Smith, G.S. and Reid, R.A. (1978) Biochem. J. 176, 1011–1014
22 Gots, R.E. and Bessman, S.P. (1974) Arch. Biochem. Biophys. 163, 7–14
23 Roos, N., Benz, R. and Brdiczka, D. (1982) Biochim. Biophys. Acta 866, 204–214
24 Fiek, C., Benz, R., Roos, N. and Brdiczka, D. (1982) Biochim. Biophys. Acta 688, 429–440
25 Scherer, B. and Klingenberg, M. (1974) Biochemistry 13, 161–170
26 Brdiczka, D. and Kolb, V. (1978) J. Ultrastruct. Res. 63, 94
27 Hollander, M. and Wolfe, D.A. (1973) Nonparameteric Statistical Methods. John Wiley, New York