POSSIBLE ROLE OF NON-BILAYER LIPIDS IN THE STRUCTURE OF MITOCHONDRIA

A FREEZE-FRACTURE ELECTRON MICROSCOPY STUDY

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The possible role of non-bilayer phospholipids on the structure of isolated rat liver mitochondria has been morphologically studied. Freshly isolated freeze-fractured mitochondria show smooth fracture faces with particles, representing the limiting membranes. The frequency and size of the particles is representative for the various membrane faces. Distinctly large particles and pits represent the attachment sites of cristae to the inner membrane. Liposome-like structures in the matrix are found upon incubation with Ca^{2+} and Mn^{2+}. At 5 mM Mn^{2+} and more, curved hexagonal (H_{II}) phase tubes are observed. Subsequent addition of 1 mM EDTA results in disappearance of the H_{II} tubes, and liposomal structures can again be seen. These findings are interpreted in terms of an Mn^{2+}-induced lamellar to H_{II} phase transition. Patchwork-like structures characterize the membranes of mitochondria, quenched from 37°C, as well as those incubated with Ca^{2+}, Mn^{2+}, Mg^{2+} and apo- or cytochrome *c*. This phenomenon is interpreted as being the result of the fracture plane, jumping from the outer to the inner limiting membrane and vice versa at sites of contact. A semi-fusion model, in which non-bilayer lipids are involved, is proposed for these contact sites.

Introduction

Mitochondrial membranes are relatively rich in phospholipids [1] that adopt the hexagonal (H_{II}) phase upon hydration at physiological temperature, pH and ionic strength. Phosphatidylethanolamine (PE) which is present in both outer and inner limiting membrane adopts the H_{II} phase in a temperature dependent way [2], whereas cardiolipin which is predominantly localized in the inner membrane [3] does so in the presence of divalent cations [4]. In mixtures with bilayer stabilizing lipids like phosphatidylcholine (PC), both PE and cardiolipin induce a variety of intermediate structures under appropriate conditions, such as lipidic particles extending into hexagonal tubes [5] and hexagonal tubes dilating into stacked bilayers [6]. Such intermediate structures have also been found in the total lipid extract of mitochondrial membranes, especially upon addition of Ca^{2+} [7].

In spite of the non-bilayer potency of mitochondrial membrane lipids, in the mitochondrion the lipids are apparently present in the bilayer configuration as is interpreted from thin section and freeze-fracture electron microscopy [8] and has recently been deduced from ³¹P-NMR [9]. However, it cannot be excluded that the non-bilayer favouring lipids express their preferential organization locally in a dynamic way. We have investigated this postulate. We have studied the morphology of freshly isolated rat liver mitochondria using freeze-fracture electron mi-

croscopy, applying the ultra-rapid jet freezing technique [10], under conditions where non-bilayer structures can be expected. Several variables, such as temperature, divalent cations and mitochondrial proteins were introduced.

We observed two morphological phenomena: First, the presence of liposomal structures in the mitochondrial matrix upon incubation with Ca^{2+}, Mn^{2+} and Mg^{2+}. Secondly, upon incubation of the mitochondrion at physiological temperature, or in the presence of (apo-)cytochrome c or divalent cations at 23°C, the membranes are fractured in a patchwork-like structure, which indicates an increased jumping of the fracture plane from outer to inner membrane and vice versa. This latter phenomenon, interpreted as an increased number of joining sites between outer and inner membrane [11], will be discussed at the molecular level.

Materials and Methods

Male Wistar rats were fasted 24 h before decapitation. Mitochondria were isolated from the liver according to the methods of Parsons and Williams [12]. Cytochrome c was purchased from Sigma (Type 6). Apocytochrome c was synthesized from cytochrome c according to Fisher et al. [13]. Incubation was performed by suspending a pellet of freshly isolated mitochondria in 1 ml of isolation buffer containing the various chloride salts or 3 mM (apo-)cytochrome c for 30 min at room temperature. Concentration series of Ca^{2+}, Mn^{2+} and Mg^{2+} were made comprising 0.25 mM, 1 mM, 5 mM, 25 mM and 100 mM. The mitochondria were concentrated prior to freezing by a brief centrifugation. The samples were jet-frozen [10] without any cryoprotectant, using the ultra-rapid jet freezer (Balzers Cryo Jet QFD-101). Mitochondria, to be quenched at 37°C, were equilibrated for 2 min at this temperature in the temperature controlled specimen holder as described before [14], and instantaneously frozen by jet freezing.
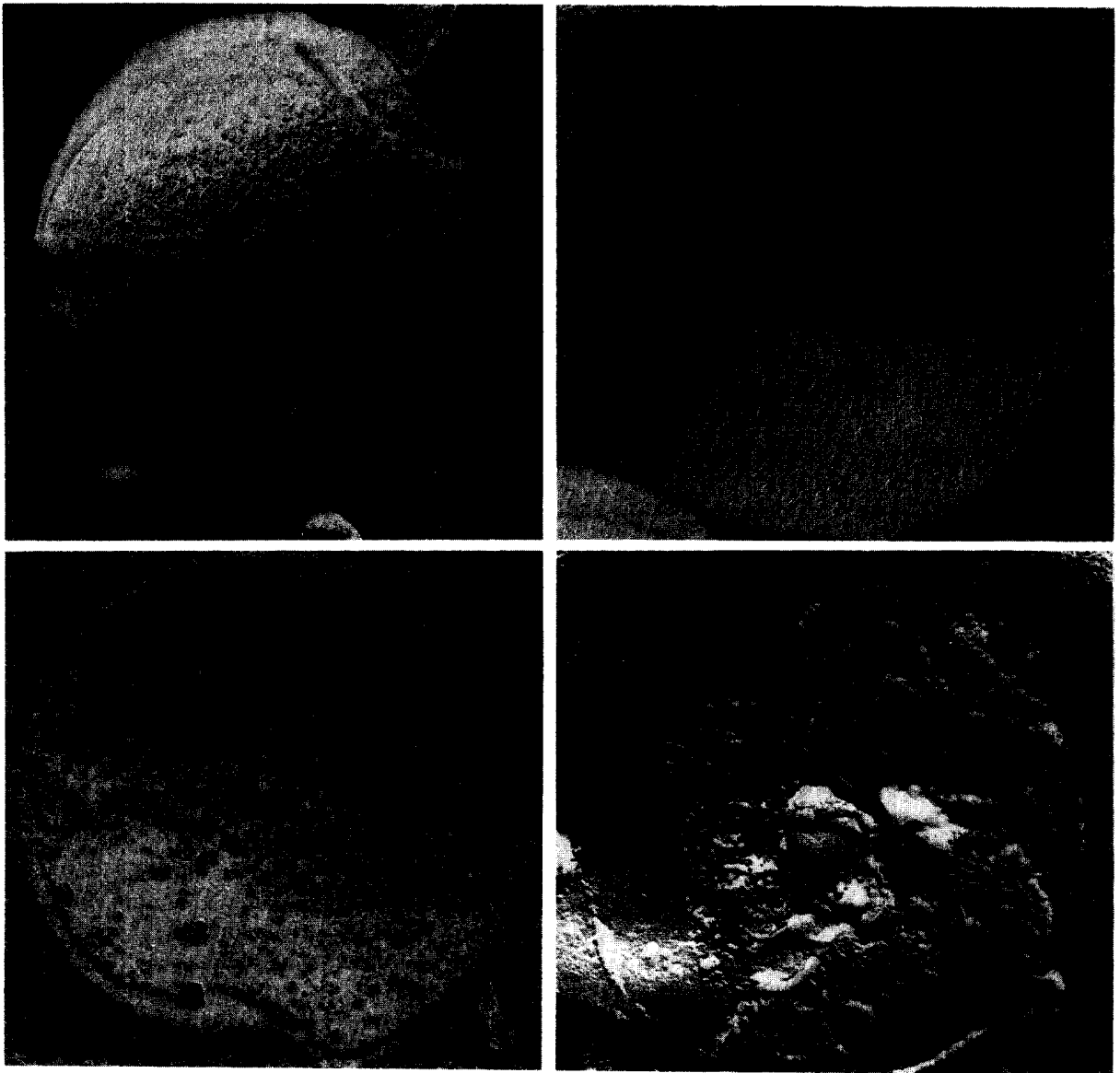
Results

Freshly isolated mitochondria were incubated for 30 min in isolation buffer. Smooth fracture faces covered with particles were observed, on both outer and inner mitochondrial membrane as has been described before [15–17]. The frequency and size of these particles is characteristic of the different fracture faces [8]. The exoplasmic fracture face (EF) of the outer membrane (OEF) contains very few and ill-defined particles (Figs. 1–2).

Larger bulges and pits are seen (diameter ≈ 25 nm) in addition to the particles ranging from 50 to 125 Å on the exoplasmic and protoplasmic fracture face of the inner membrane, respectively (Fig. 3). These structures likely represent the tubular attachment sites of cristae to the inner limiting membrane.

The first phenomenon which is evident upon incubation of mitochondria with Ca^{2+} or Mn^{2+} is the appearance of liposomal structures in the matrix of cross-fractured mitochondria (Fig. 4). These structures, induced by calcium incubation, were described before [18]. The liposomal structures were frequently associated with the inner membrane. Interestingly, incubation with 5 mM Mn^{2+} or more induces curved tubes in the liposomes (Fig. 5). These tubes are characteristic for the hexagonal H_{II} phase. These hexagonal tubes transform back to smooth fracture faces of liposomal structures upon incubation with 1 mM Na-EDTA, indicating that the induction of the H_{II} structures by Mn^{2+} is reversible.

The second and most interesting phenomenon is, that under specific conditions of incubation the fracture plane through mitochondria does not show the smooth, particulate appearance as seen in the control mitochondria (Figs. 1–3), but instead a patchwork-like structure as described before [16] can be seen. When the fracture plane jumps from outer to inner membrane and vice versa, the steps are very small in comparison with the ones seen in Figs. 1 and 2. In convex fractures, the particle frequency and size on the patches correspond to the OEF (Figs. 6, 8 and 9), whereas in concave fractures the particles on the patches are in accordance with those on the exoplasmic fracture face of the inner limiting membrane (IEF) (Fig. 7). The fractured areas beneath the patches resemble the IPF and the OPF, respectively. This patchwork-like structure is seen in mitochondria quenched from 37°C (Fig. 6) and also in mitochondria that are incubated with 1 mM or more Ca^{2+} (Fig. 7), Mn^{2+}

Figs. 1–3. Freshly isolated mitochondria, jet-frozen from 23°C.

Fig. 1. A convex fracture, displaying the exoplasmic fracture face of the outer limiting membrane (OEF) and the protoplasmic fracture face of the inner membrane (IPF) (80000 ×).

Fig. 2. A concave fracture reveals the protoplasmic fracture face of the outer membrane (OPF) and the exoplasmic fracture face of the inner membrane (IEF) (80000 ×).

Fig. 3. An IEF, with particles like in Fig. 2 and larger particles, probably representing the attachment site of cristae (80000 ×).

Fig. 4. Mitochondrion, incubated with 1 mM Mn^{2+} for 30 min at 23°C. Cross fracture through the mitochondrial matrix, containing a liposome-like structure (arrow) (100000 ×).
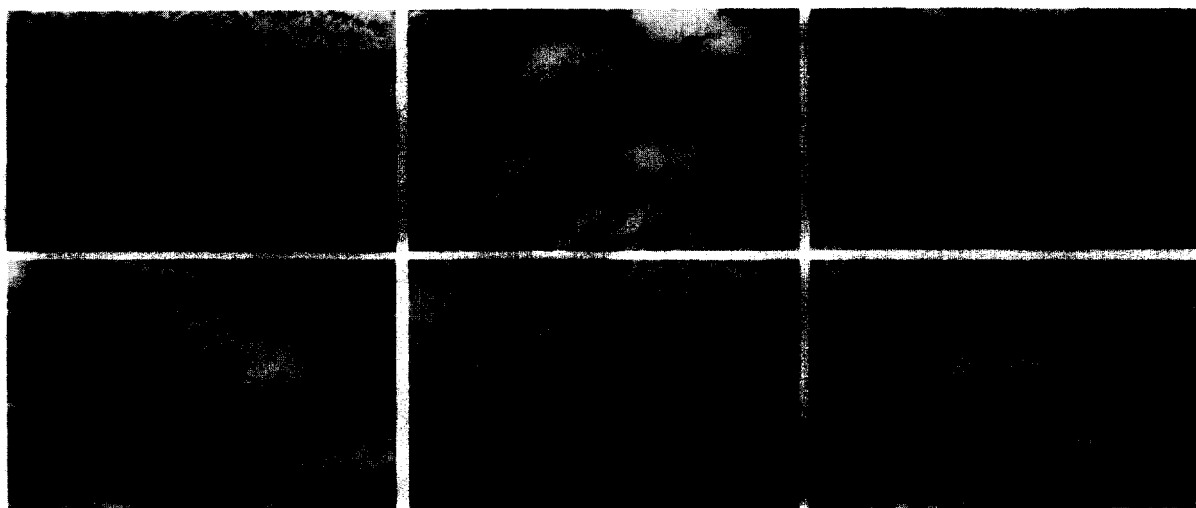
Fig. 5. Mitochondria, incubated with various concentrations Mn^{2+} for 30 min at 23°C. Structures in the matrix of mitochondria, incubated with: (a) 0.25 mM Mn^{2+}; (b) 1 mM Mn^{2+}; (c) and (d) 5 mM Mn^{2+}; (e) 25 mM Mn^{2+}; (f) 100 mM Mn^{2+}. Liposomal structures are found in (a–c). Hexagonal tube-like structures are seen in (d–f). ($100000\times$). In all micrographs the shadow direction is from below.

and Mg^{2+} (Fig. 8). Both the temperature and the divalent cation induced patches are reversible. Incubation at 37°C for 25 min, followed by cooling to 20°C, or incubation with 25 mM Mn^{2+} and subsequent incubation with 1 mM Na-EDTA resulted in smooth, patchwork-free fracture faces. Mg^{2+} shows a tendency to aggregate the intramembranous particles into clusters. With Ca^{2+} or Mn^{2+}, this phenomenon was rarely found. Cytochrome c as well as its biological precursor apocytochrome c (Fig. 9) also induce patchwork structures.
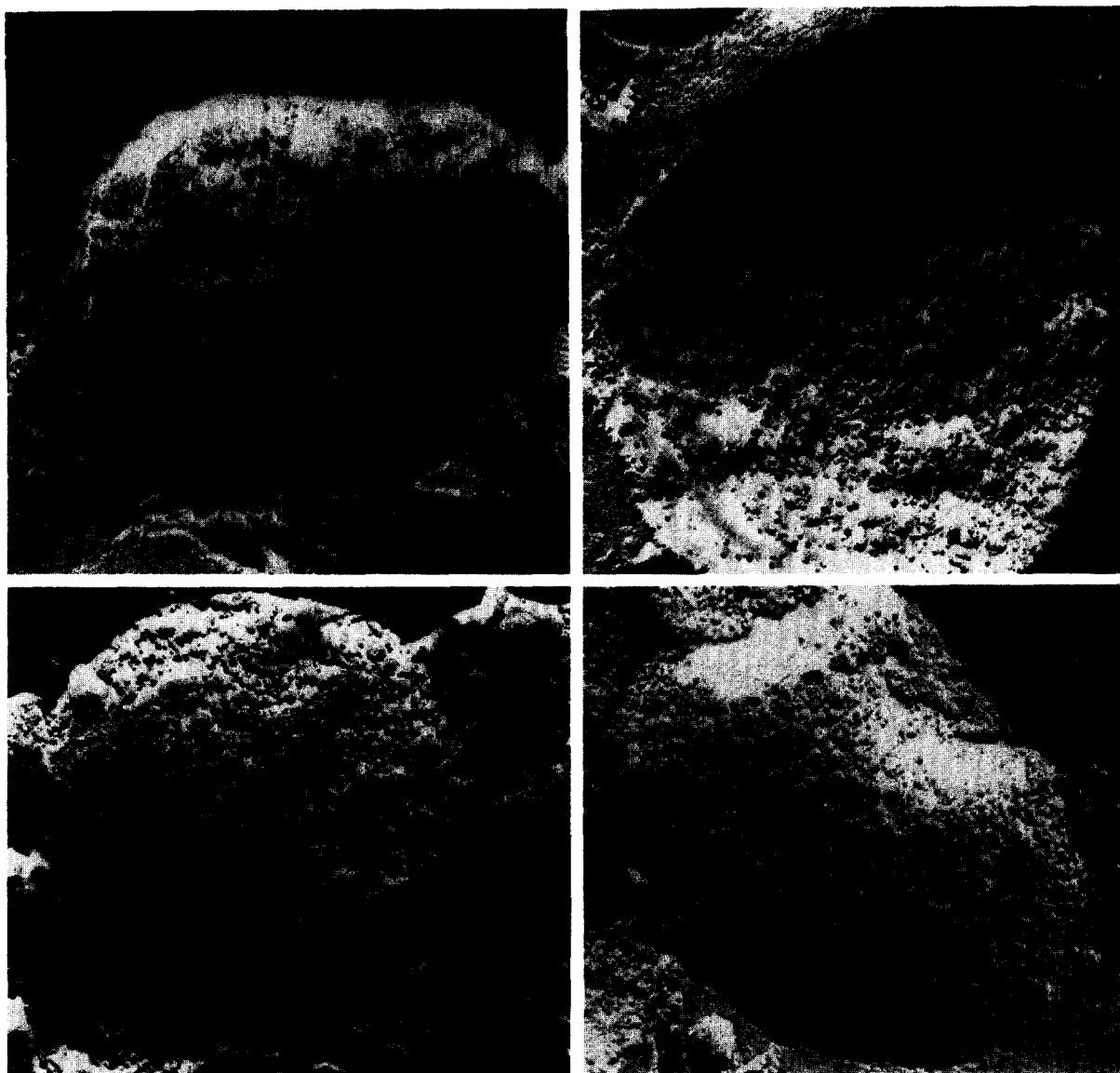
In order to assess the possibility that the patches appear as a result of non-specific swelling of the mitochondrial matrix, causing a close apposition of the two limiting membranes thus creating a situation in which the fracture can jump easily from outer to inner membrane and vice versa, we studied mitochondria in hypotonic sucrose buffers. Mitochondria, incubated in 100 mM sucrose were found to rarely exhibit patches. These findings indicate that swelling per sé does not induce patches.

Discussion

Mitochondrial membranes, rich in non-bilayer favouring phospholipids, are quite appealing in the search for polymorphic lipid structures in biological membranes. In this paper we present evidence that mitochondrial lipids express their non-bilayer organizations which may play a role in mitochondrial structure and physiology.

The liposomal structures seen in the mitochondrial matrix in the presence of Ca^{2+} or Mn^{2+} can be converted into hexagonal (H_{II}) phase tubes, upon incubation with 5 mM Mn^{2+} or more. It can be postulated that Ca^{2+} and Mn^{2+} can induce some non-bilayer structures as a consequence of electrostatic interaction with negatively charged lipids, e.g. cardiolipin. When the concentration of the divalent cations exceeds a certain value, the formation of non-bilayer structures is followed by a structural phase separation with resultant pinching off of lipids into the matrix. These liposomal structures are enriched in cardiolipin, as determined in isolated matrix granules [19]. Moreover, with the aid of electron microscopic elemental analysis, Ca^{2+} could be detected inside these structures [20]. The liposomes are devoid of intramembraneous particles (IMP's). This morphology is expected in the light of the small amount of protein which is known to exist in matrix granules [20]. Apparently, Mn^{2+} has the capacity to induce a transition from the lamellar to H_{II} phase in these structures in contrast to Ca^{2+}.

Figs. 6–9. Patchwork-like structures in mitochondrial membranes (100000 ×).

Fig. 6. A convex facture through a mitochondrion, quenched from 37°C. Patches (arrows) of the outer membrane are seen on top of the inner membrane. Note the small step when compared to mitochondria at 23°C (Fig. 1).

Fig. 7. A concave fracture through a mitochondrion, incubated for 30 min at 23°C in 5 mM Ca^{2+}, displaying patches (arrow) of the inner membrane superimposed on the outer membrane.

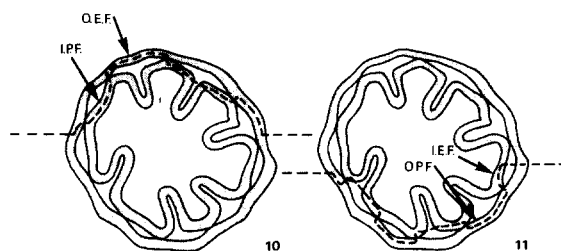Fig. 8. A mitochondrion, incubated in 25 mM Mg^{2+}.

Fig. 9. A mitochondrion, incubated in 3 mM apocytochrome c.

This is in variance with lipid model systems where both Ca^{2+} and Mn^{2+} induce H_{II} phase in pure cardiolipin liposomes [4,6]. Speculating about the functional meaning of this result, one might suggest that these liposomal structures are Ca^{2+} and Mn^{2+} sequestering sites as it is well known that mitochondria can actively accumulate these ions. However, it is still an open question whether in vitro incubation with ion concentrations as high as 5 mM, has any physiological importance. In vivo, mitochondria accumulate Ca^{2+} also in calcium phosphate granules, even in preference over Ca-membrane storage [18]. Barnard and co-workers [20] presented an alternative hypothesis. They suggest that the liposomal matrix granules are precursors of the inner mitochondrial membrane.

The patchwork structures have been described before [11,16,17]. We agree with the interpretation of these authors that the patches are pieces of one limiting membrane, lying on the other membrane. This is supported by our finding that the particle densities and sizes of the patches are similar with respect to the freshly isolated mitochondrial membranes. Apparently, during the fracturing process the fracture plane jumps from the outer membrane to the inner and back (Figs. 10 and 11).

It may be argued, that the fracture plane jumps at sites of close apposition (not contact) between the two membranes (Fig. 12a). Matrix swelling due to accumulation of cations or incubation at elevated temperature [21] will minimize the space between outer and inner limiting membrane, thus facilitating fracture plane jumping. However, our experiments with mitochondria swollen in hypotonic sucrose buffer do not exhibit patches. Therefore, it is reasonable to assume that the loci of fracture plane jumping are real contact sites as also suggested by Brdiczka [11]. Contact sites have been described before in thin sections of isolated rat liver mitochondria [22].

Our results with divalent cations may thus firstly be explained by proposing the formation of contact sites due to adhesion between the two apposing membranes (Fig. 12b). In that case, there are only polar interactions between the phospholipids as a result of charge neutralization and dehydration [4]. It can also be suggested that the apposing monolayers of the two membranes fuse (Figs. 12c and 12d). Then, the fusion process which is half-

Figs. 10 and 11. The rationale for the formation of patchwork-like structures in mitochondrial membranes. The fracture plane jumps from the outer limiting membrane to the inner and vice versa at sites of contact.

Fig. 10. A convex fracture reveals the exoplasmic fracture face of the outer membrane (OEF) and the protoplasmic fracture face of the inner membrane (IPF) (compare with Figs. 6, 8 and 9).

Fig. 11. A concave fracture reveals the protoplasmic fracture face of the outer membrane (OPF) and the exoplasmic fracture face of the inner membrane (IEF).

way complete is arrested and a subsequent fission does not occur. This semi-fusion or joining model for mitochondrial contact sites is favoured over the adhesion idea by the results of the 37°C experiments. The formation of patches at 37°C cannot be explained by adhesion as charge neutraliza-
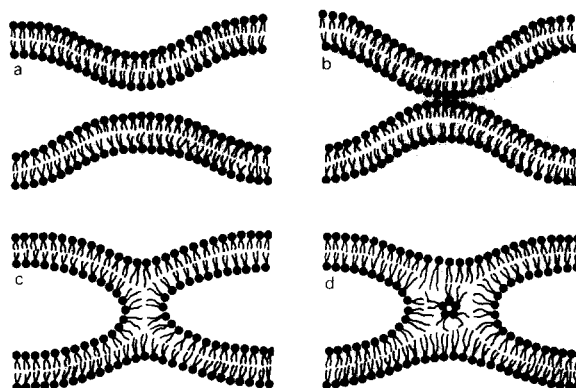

Fig. 12. Four models, depicting the possible molecular interpretation of the site of fracture plane jumping, shown in Figs. 6–9. (a) Close apposition of the inner and outer membrane. (b) Contact between the two membranes without disruption of the bilayers (adhesion). (c) and (d) Semi-fusion, (c) without, and (d) with an inverted micelle or H_{II}-tube at the nexus. The two apposing monolayers fuse; the other monolayers remain intact.

tion does not occur. We think that some mitochondrial lipids (e.g. PE) feature a lamellar to hexagonal (H_{II}) phase transition upon heating the mitochondria from 20°C.

This semi-fusion model can be envisaged in two alternative ways: apolar contact between the apposing monolayers with (Fig. 12d), or without (Fig. 12c) an inverted micelle or extended small inverted hexagonal H_{II} cylinder at the nexus of the two bilayers. The latter model has recently been proposed for the molecular interpretation of tight-junctions although in that model two cylinders were proposed instead of one [23]. We will discuss both models without having the intention to favour one of them.

All experiments presented here suggest some involvement of non-bilayer lipids in the formation of these semi-fusion (joining) sites. These non-bilayer lipids can facilitate the formation of highly curved monolayers at the joining site (Figs. 12c and 12d) and they may also be involved in inverted micelle or H_{II} cylinder (Fig. 12d) formation. The results of the incubation experiments at 37°C strongly support the involvement of PE which

adopts the hexagonal H_{II} phase at this physiological temperature. The effect of the divalent cations may be explained as follows: firstly, cardiolipin adopts the H_{II} phase upon addition of various divalent cations [4,6]. Secondly, the presence of divalent cations may facilitate the expression of non-bilayer configurations of PE. This is supported by the fact that in the cardiolipin/phosphatidylethanolamine system, cardiolipin stabilizes the bilayer in the absence of Ca^{2+} [24]. Addition of Ca^{2+} induces a total transition to the H_{II} phase. Probably, cardiolipin is withdrawn from its interaction with PE by the complexation with Ca^{2+}. The effects of both cytochrome c and apocytochrome c can be explained in a similar manner since both proteins induce changes in the molecular organization of cardiolipin systems, as found with ^{31}P-NMR and freeze-fracture electron microscopy (Ref. 24; De Kruijff, B., unpublished data). In this respect it is interesting to note that also hydrated mitochondrial lipid extracts organize in non-bilayer structures upon addition of Ca^{2+} [7].

One could argue, that the morphology of mitochondria as presented here favours the model

Fig. 13. Tight junction in epithelium of rat ileum. Fracture planes, jumping from one bilayer to another at contact sites, display steps (arrows), but no lipidic particles or ridges (100000×).

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without the inverted micelle (Fig. 12c) because the patch edges only display a step in contrast to lipid model systems and tight-junctions where particles and complementary pits as well as ridges and complementary fissures are encoutered. However, it is possible that an inverted micelle or cylinder is present (Fig. 12d), but not visible. Apparently in mitochondria for some unknown reason the fracture plane tends to jump from one membrane to the other at the joining points. When the fracture plane jumps in this fashion in lipid model systems, which is rarely observed, recognition of lipidic particles is difficult (Ref. 25: Fig. 1i). Recognition of individual hexagonal tubes when the fracture jumps from one membrane to the other is even harder than in the case of lipidic particles as is illustrated in micrographs obtained from tight-junctions of rat ileum epithelial cells (Fig. 13). Recognition of H_{II} rods is harder than of inverted micelles, probably because H_{II} rods are mostly somewhat smaller than the corresponding inverted micelles [6]. Moreover, the observation of a step at the patch edge would be very difficult, if not impossible, in the absence of an inverted micellar or cylindrical structure (compare Figs. 12c and 12d).

With respect to the proposed presence of an inverted micelle or cylinder in the joining site (Fig. 12d), the following can be noted. It is found in model systems, that in the absence of ethylene glycol H_{II} cylinders are preferentially observed as compared to lipidic particles [26]. The presence of a H_{II} cylinder rather than an inverted micelle at the mitochondrial joining site is feasable, since a hydrated non-glycerinated suspension of isolated inner mitochondrial membrane lipids reveals ridges in preference over lipidic particles (Van Venetië, R., unpublished data). These ridges are reminiscent of those found in the PC/PE/cholesterol [27] and the mixed galactosyldiacylglycerol [28] systems.

The joining site model, in which lipids can easily diffuse from outer to inner membrane, can explain the accelerated transport of cytoplasmically synthesized PC from the outer to the inner mitochondrial membrane upon perfusion of rat livers with 18 mM calcium acetate [29], when this is correlated to our finding that Ca^{2+} incubation of mitochondria in vitro induces patches. In a model membrane system of phosphatidylcholine/cardiolipin, addition of Ca^{2+} increases the flip-flop rate of PC [30].

In conclusion, the semi-fusion model for mitochondrial contact sites is quite attractive on basis of our experiments. The nexus of the joining sites is of a lipidic nature. However, we are not able to discriminate between the two alternative joining models (Figs. 12c and 12d).

It is tempting to speculate about the physiological implications of semi-fusion sites. Transport of various molecules between cytoplasm and matrix is much facilitated when carried out across semi-fusion sites [22]. ATP-ADP translocase may be associated with contact sites; its activity, high in state 3 and low in state 4, being explained by the presence or absence of contact sites [31]. The import of mitochondrial proteins, synthesized on cytoplasmic ribosomes, may be mediated by contact sites. Our experiments with cytochrome c and its precursor apocytochrome c illustrate this proposal. Recently, the involvement of contact sites in the import of cytochrome b and cytochrome c_1, was suggested [32]. As discussed above, the transport of phosphatidylcholine from the outer to the inner mitochondrial membrane [29] may be mediated by contact sites.

In conclusion, this paper supports the relevance of lipid polymorphism in isolated mitochondria, in that it may be involved in the formation of semi-fusion sites between the two limiting membranes.

Acknowledgements

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