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The Ribbon Structure of the Mitochondrial Inner Membrane

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Abstract

Electron microscopic evidence is presented for the extensive association of protein subunits into ribbons within the mitochondrial inner membrane. The mitochondrial cristae can be rearranged to a narrow tubular form which exhibits ribbon structure and is fully functional; the morphology of particles derived from sub-mitochondrial electron transport particles by treatment with lysolecithin suggests that the backbone of the ribbon is provided by the cytochrome-free tripartite unit (headpiece, stalk, basepiece) in linear repeat. These results are inconsistent with any single model of the inner membrane previously proposed, but are best understood in terms of a model which combines the concept of an ordered protein continuum with the concept of a fluid lipid bilayer. Further, it is concluded that the "headpiece out" morphology of the tripartite unit represents a viable conformation of the endergonic transducing unit.

Introduction

Any understanding of mitochondrial energy transduction must eventually encompass known features of the ultrastructural arrangement of the transducing units. Knowledge of this arrangement is therefore highly desirable.

Previously, proposals have ranged from highly ordered protein continua [1,2] to individual protein units free to diffuse at random in a fluid lipid bilayer [3], views arrived at from different experimental and theoretical standpoints. In this paper, a structure of the mitochondrial inner membrane is proposed which reconciles these apparently opposite concepts.

The stimulus for our model arose, however, from experimental rather than theoretical considerations. While studying the morphology of non-membranous particles derived from ETP* by the action of lysolecithin, we became convinced that their essential structure was that of a ribbon of TRU.† This led us to search for evidence that ribbon structure is a property of the intact membrane and not just artifactual. We have found such evidence in a study of hypotonically swollen mitochondria and particles derived therefrom. Although our own observations are limited to the mitochondrial inner membrane, a considerable body of evidence suggests that the concept of ribbon structure has much wider application.

Methods

Tubular Cristae and ETP

Heavy beef heart mitochondria were prepared by the method of Hatefi and Lester [4] and stored in a deep freeze at a concentration of 50 mg/ml. Mitochondria frozen for 1 to 10 days were used, better activities being retained by shorter freezing time. Frozen mitochondria, 20 ml, were warmed to 30°C and held at this temperature for 1 min before being added to 400 ml 10 mM Tris-Cl, pH 7.8, at 22°C. After 5 min, the suspension was centrifuged for 10 min at 27,000 × *g*. with refrigeration. The pellets were cooled at 4°C for 2 hr; the fluffy layer was resuspended to 100 ml with ice-cold 10 mM Tris-Cl. This suspension consisted of swollen mitochondria containing tubular cristae. Tubular ETP was prepared from this by sonication at 0°C with a Branson Sonifier, Model W185, in 50-ml batches for 6 sec with output control setting 6 (about 60 W output). The sonicated suspension was centrifuged for 10 min at 27,000 × *g*, and the supernatant collected. This supernatant was centrifuged once for 10 min at 50,000 × *g* to yield pellets designated "heavy tubular ETP," and again for 10 min at 50,000 × *g* to yield pellets designated "light tubular ETP." Pellets were resuspended by homogenization to approximately 10 mg/ml in

* ETP: electron transport particle, an inner membrane particle derived from mitochondria by sonication and differential ultracentrifugation.

† TRU: tripartite repeating unit, i.e., headpiece (F_1), stalk (oligomycin sensitivity conferring protein), and basepiece.

10 mM Tris-Cl, pH 8.2, at 0°C. The control ETP was prepared by exactly the same procedure except that 250 mM sucrose was incorporated in the buffer throughout.

Lysolecithin Treatment

ATP–P_i exchangease particles were prepared from ETP by treatment with lysolecithin, and by differential centrifugation as previously described [5]. Asolectin vesicles were prepared by homogenization of 5 g asolectin in 100 ml 10 mM Tris-Cl, 1 mM EDTA, pH 8.0, followed by sonication at 0°C for 5 min.

Electron Microscopy

(a) *Negative Staining.* The suspension of swollen mitochondria was diluted to approximately 0.3 mg/ml in 10 mM Tris-Cl, pH 8.0, and a drop of dilute suspension was applied to a grid. Excess sample was removed, and the residue was stained with 1.3% ammonium molybdate, pH 7.5, and examined in a Hitachi 11E electron microscope. Other samples were diluted to 1 mg/ml and prepared similarly, after fixation for 5 min by 0.1% glutaraldehyde.
(b) *Thin-Sectioning.* A suspension of swollen mitochondria was fixed by addition of an equal volume of cold 2% glutaraldehyde in 10 mM Tris-Cl, pH 8.0. The procedure thereafter was as previously described [6], except that 10 mM Tris-Cl, pH 8.0, was used as the buffer throughout.
(c) *Freeze-Fracture.* Specimens were prepared by standard procedures as described [7], except that swollen mitochondria in 30% glycerol were centrifuged for 10 min at 27,000 × *g* before being frozen in Freon 22.

Biochemical Assays

Oxygen uptake was measured at 30°C using a Beckman oxygen analyzer. For the determination of respiratory control, the reaction mixture (3 ml) contained 250 mM sucrose, 20 mM Tris-Cl, pH 7.4, and 2-mg particles. Thirty microliters of 100 mM NADH were added to start the reaction, followed after 1 min by 1 μM carbonylcyanide *m*-chlorophenylhydrazone (mClCCP). The oxidative phosphorylation assay mixture (5 ml) contained 250 mM sucrose; 20 mM Tris-Cl, pH 7.4; 3 mM $MgCl_2$; 1 mM ADP; 4 mM potassium phosphate containing $^{32}P_i$ (4×10^5 cpm/μmole); 5 mM glucose; 20 units/ml hexokinase; and 2-mg particles. Phosphorylation was initiated by the addition of 40 μl 100 mM NADH. The procedure thereafter was as described earlier [12]. ATP–P_i exchange activity was assayed as described

earlier [5], except that 10 mM KCl was incorporated into the reaction medium. Cytochrome *c* oxidase activity was measured in a medium containing 250 mM sucrose; 20 mM Tris-Cl, pH 7.4, at 30°C; 1.8 mg cytochrome *c* (type III from horse heart, Sigma Chemical Company); 0.1-

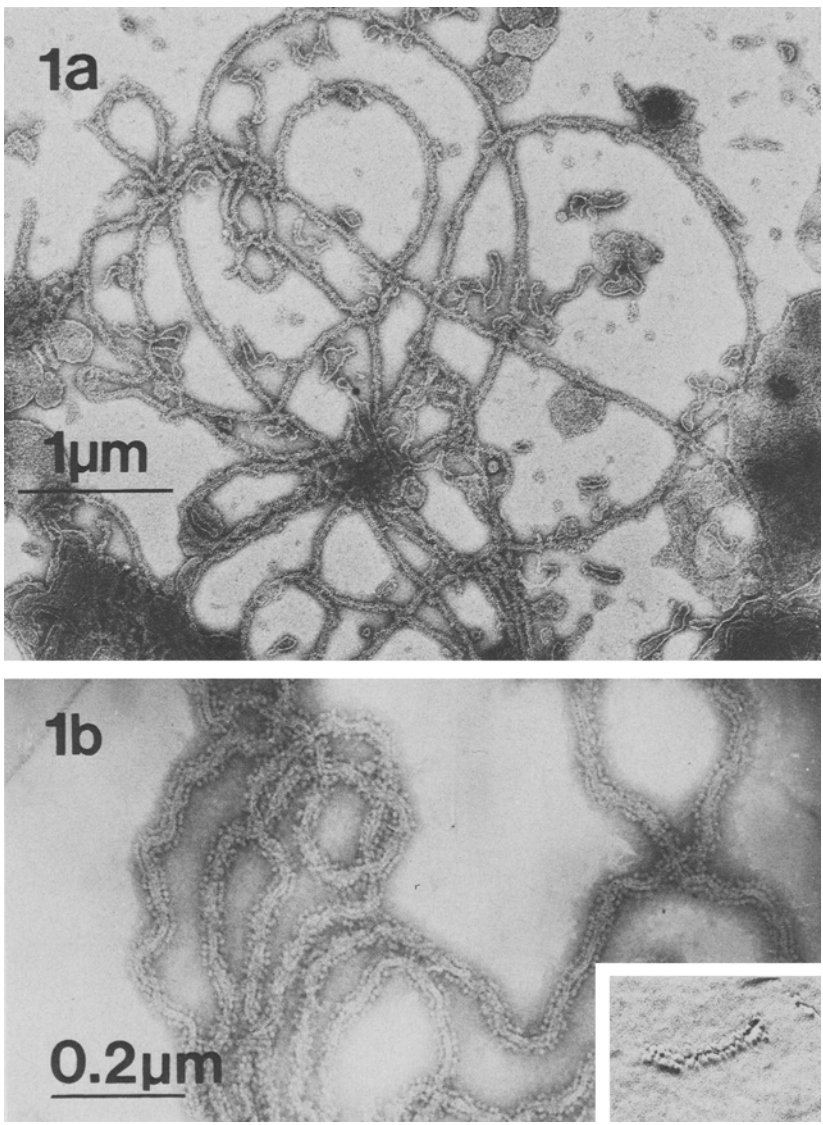

Figure 1. Tubular cristae of swollen mitochondria as visualized by negative staining. (a) ×23,500; (b) ×85,000. Inset: light tubular ETP, as visualized by freeze-fracture.

or 0.2-mg particles; and 0.1 or 0.2 mg lysolecithin in a total volume of 2.7 ml. Respiration was measured polarographically on the addition of 0.3 ml 500 mM potassium ascorbate, pH 7.4.

Lipid-Depleted Mitochondria

These were prepared by the method of Fleischer et al. [9], except that after lipid extraction, the mitochondria were fixed for 1 hr in a solution 1% in glutaraldehyde, 50 mM in cacodylate (pH 7.4 at 0°C), and 250 mM in sucrose, and then washed for 1 hr in the same buffer. The procedure thereafter was as described earlier [6]. The extent of lipid depletion was estimated by measuring the phosphorus [10] and protein contents [11] of the extracted mitochondria.

Results and Discussion

The process of swelling beef heart mitochondria under the conditions described (see Methods) causes reorganization of the cristae to a form we interpret as being tubular, from their appearance by negative staining (Fig. 1) and in thin section (Fig. 2). In negatively stained preparations, each reorganized crista exhibits two parallel rows of TRU. In thin section, a circular cross-section is observed, indicating that the TRU ribbons are part of the wall of a tube, as illustrated in Fig. 3a. The tubes are seen to be bounded by (and are presumably attached to) a part of the inner membrane which has ballooned out on swelling. The original orientation of the membrane with respect to the bathing solution is thus retained (Fig. 3b). This is borne out by the observation of proton ejection in response to energization (data not shown). The balloon part of the membrane is ruptured during drying down to give the disemboweled appearance usually seen in negatively stained preparations (Fig. 1).

Investigation of the properties of the tubes has been facilitated by their enrichment in the "tubular ETP" fractions (Figs. 4a and 4b), derived from the swollen mitochondria by sonication and differential centrifugation (see Methods). The light tubular ETP fraction was found to contain the highest proportion of tubes (Fig. 4b). The properties of tubular ETP are similar to those of control ETP which consists of round vesicles (Table I, Fig. 4c).

The question arises as to whether the ends of the tubes are open or sealed. The particles are well coupled (Table I), but in this laboratory we have previously found conditions under which coupling is retained in non-membranous particles [12]. Information about membrane integrity in tubular ETP can be gained by the response of ATP–P_i exchange activity to the action of valinomycin plus nigericin in the presence of potassium ions.

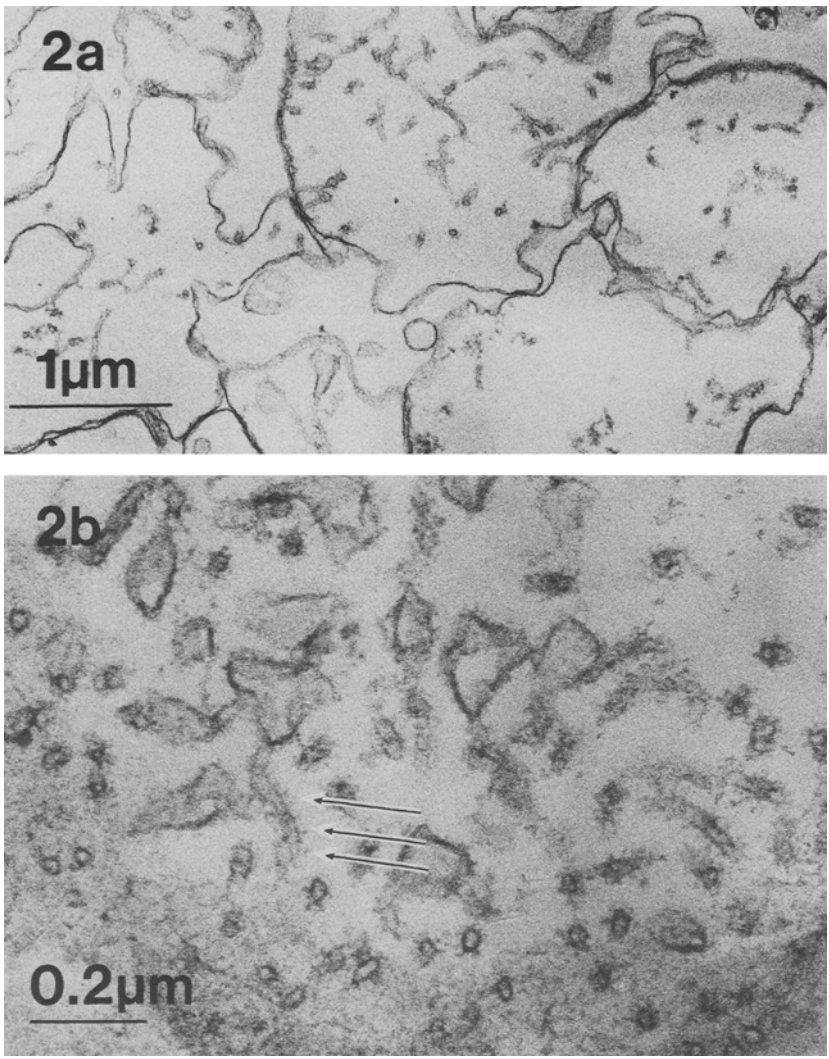

Figure 2. Tubular cristae of swollen mitochondria as visualized in thin sections. (a) ×23,500; (b) ×85,000.

In response to ATP hydrolysis, valinomycin will mediate the transport of K^+ out of an ETP vesicle as a positively charged valinomycin–K^+ complex. The process is immediately inhibited, however, by depletion of K^+ from the internal aqueous phase and the buildup of H^+ [13]. The addition of nigericin collapses these gradients, since nigericin is able to take in K^+ as

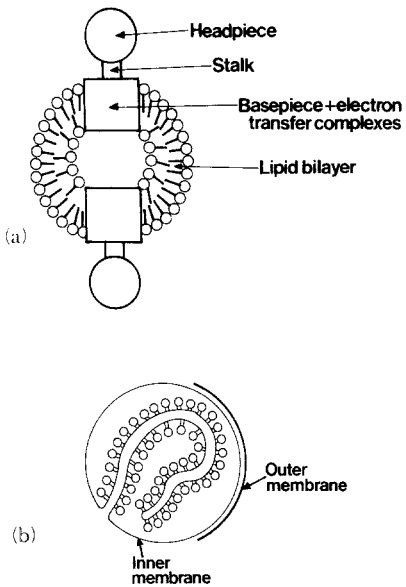

Figure 3. The proposed structure of a tubular crista. (a) Cross-section; (b) longitudinal section.

TABLE I. A Comparison of Activities of Control and Tubular ETP[a]

	Heavy T ETP	Light T ETP	Heavy C ETP	Light C ETP
ATP–P_i exchange (nmole/min/mg)	117	147	103	94
+ valinomycin (2 μg/mg)		139		
+ nigericin (2 μg/mg)		82		
+ valinomycin + nigericin		2		
P/O (NADH)	1.36	1.90	1.25	1.31
nmole P_i esterified/min/mg	148	117	186	156
NADH oxidase (units/mg)	0.30	0.17	0.43	0.32
+ 1 μM mCl-CCP	0.88	0.61	0.87	0.79
Uncoupler RCI	2.9	3.64	2.0	2.44
Cytochrome *c* oxidase (units/mg)	4.94	3.90	4.30	3.52

[a] Measurements were made on the preparations shown in Fig. 4.

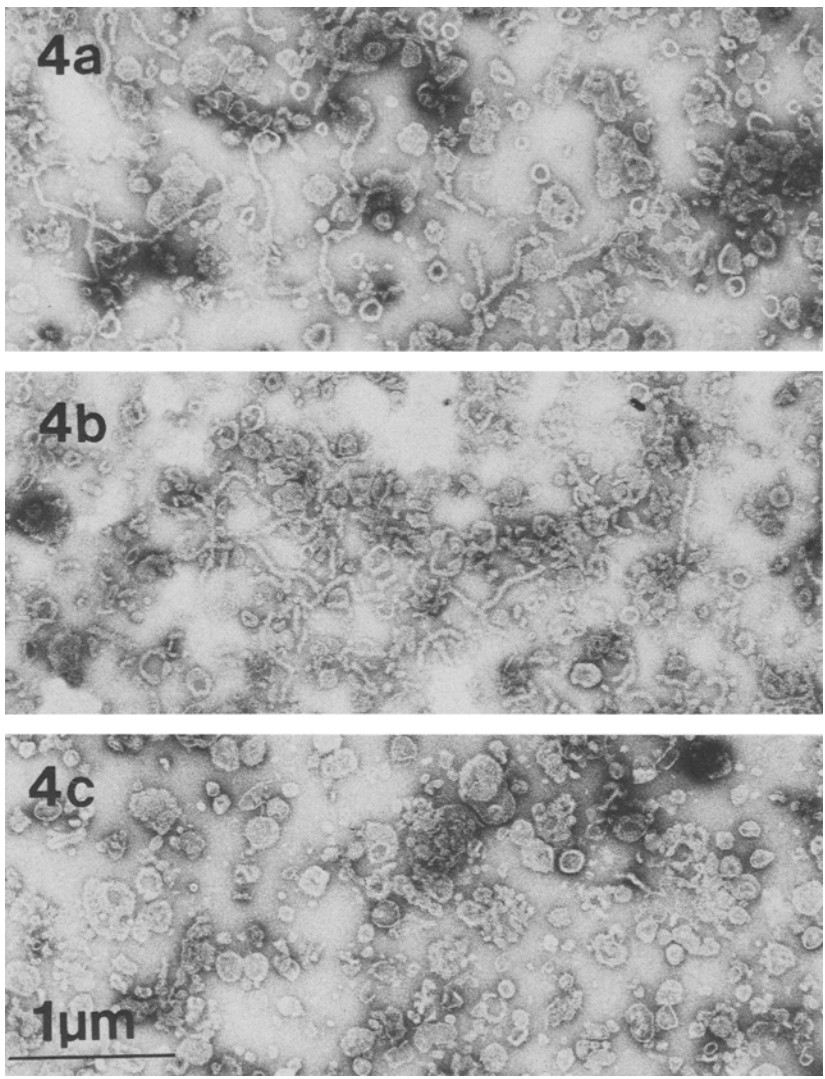

Figure 4. (a) Heavy tubular ETP; (b) light tubular ETP; (c) light control ETP. ×23,500.

an electroneutral complex and bring out H^+ in exchange. The net effect is the coupling of ATP hydrolysis to the cycling of ions, thereby preventing ATP synthesis and inhibiting ATP–P_i exchange activity. Neither ionophore alone is able to do this, and it is evident that the rationale for their synergistic action requires the presence of an internal aqueous

compartment separated from the external aqueous phase by an ion-impermeable membrane. ATP–P_i exchange activity in the tubular ETP is seen to be synergistically inhibited by the action of valinomycin plus nigericin in the presence of K^+, and therefore the ends of the tubes must be sealed, enclosing an internal aqueous phase.

The tubular ETP has consistently been found to show a higher respiratory control index and a better phosphorylating capacity than the control ETP, often surpassing that found in ETP_H. This observation is surprising, since the only difference in preparative procedure is a hypotonic swelling step at room temperature, a treatment usually considered more harmful than helpful for the preservation of oxidative phosphorylation. It is not yet clear whether the difference in coupling relates to the tubular structure *per se* or to the hypotonic treatment needed to form the tubes. The better coupling of the light ribbon ETP fraction compared with the heavy ribbon ETP fraction suggests that the former may be the case.

Further insight into the structure of the ribbons seen in tubular ETP can be gained by fragmentation of the membrane with lysolecithin. It was shown previously [5] that a particulate fraction deficient in electron transport components but possessing ATP–P_i exchange activity ("exchangease") could be derived from ETP by treatment with lysolecithin and differential centrifugation. When examined by negative staining, a particle appears as a linear array of TRU, similar in appearance to those seen in tubular cristae, usually folded over on itself to form a closed loop (Fig. 5a).

Do these loops represent a side view of vesicles, or are they linear structures in their own right? The synergistic inhibition of exchange activity by valinomycin–nigericin–K^+ indicates that small vesicles are indeed present [5]. However, linear arrays are commonly seen which are not closed, and these are often totally devoid of any structure which is remotely vesicular (Fig. 5a, arrows and inset). Such particles reveal the essential structure of the exchangease as being a ribbon of TRU. This conclusion is borne out by the effect of the addition of more lysolecithin: The appearance of the particles by negative staining is hardly changed (Fig. 5b), but the exchange activity is no longer synergistically inhibited by valinomycin–nigericin–K^+ [5]. There is therefore no indication that vesicular structure is present after treatment with more lysolecithin, and yet the ribbon structure persists. The autonomy of the ribbon is further demonstrated by incorporating it into multilamellar vesicles of asolectin phospholipid (Fig. 5c). The TRU ribbons do not disperse into single units distributed over the vesicle, but remain in discrete segments of ribbon. Exchange activity is enhanced by the addition of lipid, and synergistic inhibition by valinomycin–nigericin–K^+ is regained (data not shown). We

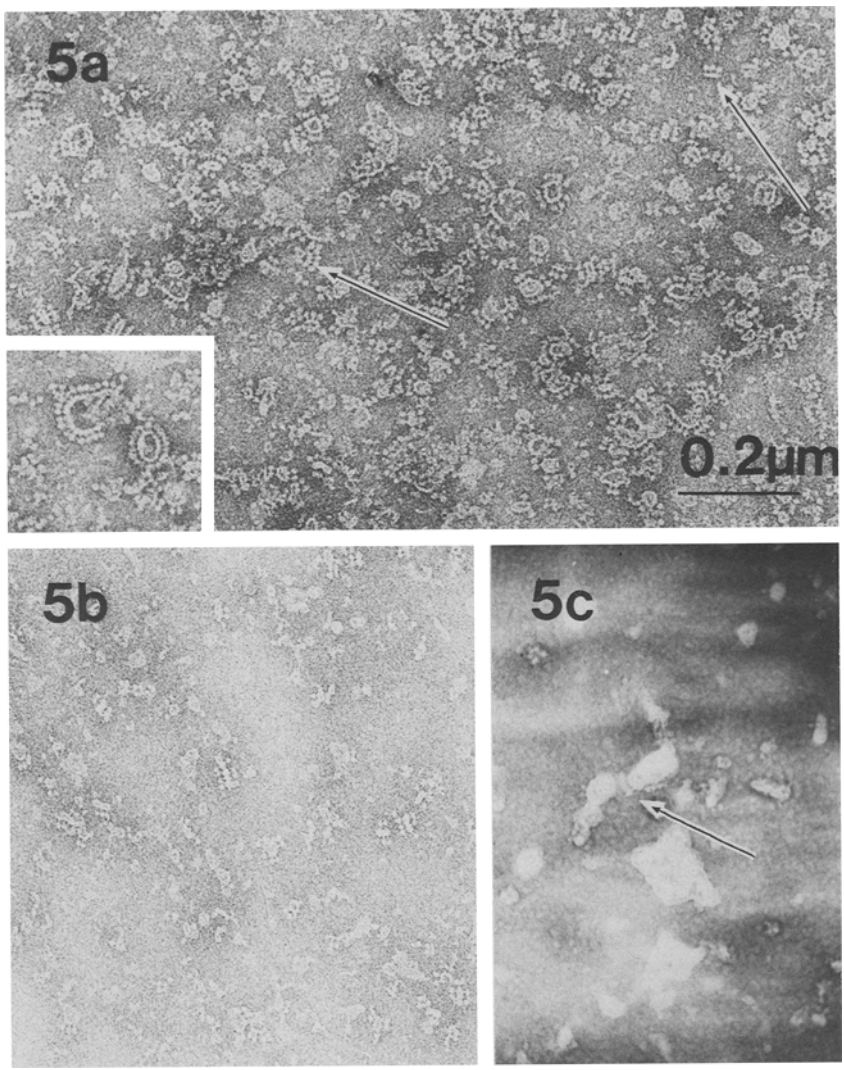

Figure 5. "ATP–P_i exchangease" particles derived from ETP by fragmentation with lysolecithin and differential centrifugation. (a) As isolated; (b) plus more lysolecithin (1.2 mg/mg protein); (c) plus 50 mg asolectin/mg exchangease. Arrows and inset indicate particles which clearly show ribbon structure. × 85,000.

are thus led to conclude that the ribbons of TRU are structures in their own right, irrespective of whether or not they are incorporated into vesicles.

The importance of these observations of the exchangease particle lies in the fact that the particles are 90% deficient in electron transfer complexes [5] and hence the backbone of the ribbon structure can be identified with the basepiece of the TRU. In the intact membrane, the electron transfer complexes presumably interact with this ribbon in the plane of the membrane, an interaction disrupted by lysolecithin.

How large is the basepiece? By negative staining, the depth of the basepiece is seen to be approximately 60 Å, suggestive of a transmembrane location in the normal membrane. The synergistic inhibition of exchange activity by valinomycin–nigericin–K^+ is further evidence for this, since the mechanism of this inhibition involves the transmembrane movement of ions. An estimate of the length of the basepiece along the ribbon is easily achieved, since it must be the same as the headpiece repeat distance. This is approximately 100 Å, in good agreement with the headpiece spacing measured in the intact membrane [14]. It is difficult to estimate the third dimension of the basepiece from negatively stained preparations of exchangease, since the exchangease ribbons tend to lie on their side on the grid. Preliminary results with freeze-fractured preparations of exchangease indicate that the width may be as narrow as 20 Å.

Which proteins comprise the basepiece ribbon? SDS gel electrophoresis of exchangease [5] shows a pattern similar to that of the oligomycin-sensitive ATPase [15], whose major components include proteins of molecular weight 29,000, 20,000, and 10,000 in addition to proteins which make up the headpiece–stalk. Treatment of the oligomycin-sensitive ATPase complex with NaBr solubilizes the headpiece proteins, but fails to remove the 29,000, 20,000, and 10,000 molecular weight components, indicating that the latter are integral to the membrane-forming portion of the complex [16]. Since exchangease also contains these components (in fact, the 29,000 component is enriched), we conclude that these proteins comprise the basepiece of the TRU.

The question arises regarding the degree to which ribbon structure is native to the usual "two-dimensional" membrane. A skeptic might argue that the ribbon structures seen after treatment with lysolecithin are artifactual, indicating only the tendency of solubilized lipoproteins to associate. Another might dismiss the ribbons seen in tubular cristae as being only a product of artificial tubular constraints. But the observation of similar ribbons in both situations renders each objection unlikely, and points to the existence within the native membrane of ribbon structure from which the lysolecithin ribbons and tubular ribbons are derived.

Such structure has in fact been observed. Crane et al. have observed ribbon structures in negatively stained cristae [17], and linear arrays of particles have been observed in the freeze-fracture studies of

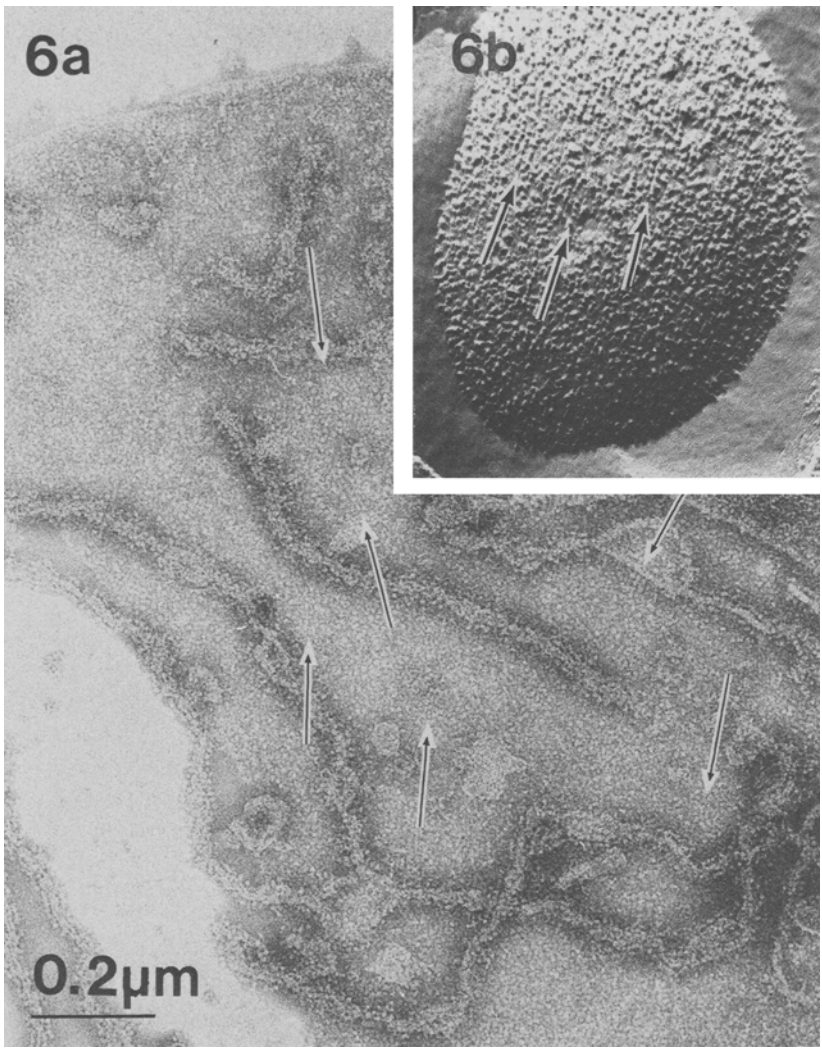

Figure 6. Part of the inner membrane which balloons out on swelling. (a) By negative staining; (b) by freeze-fracture. Ribbon structure is best seen by viewing along the arrows. ×85,000.

Wrigglesworth et al. [18] and Hackenbrock [19]. We also find that ribbon structure is commonly observable in the inner membrane, but that it is often not apparent at first glance. The reason is that the ribbon structure does not impose long-range order on the membrane, but rather gives the membrane the appearance of being lightly combed. An example is given in

Fig. 6, which shows the ballooned-out part of the swollen inner membrane as seen by negative staining and by freeze-fracture. The ribbon structure is most evident when viewed along the direction of the arrows. On careful examination, rows of subunits are clearly visible, with rows often in register. Rows of subunits are sometimes curved, suggesting that the ribbons have a certain degree of flexibility in the plane of the membrane. This appearance is entirely consistent with the notion that ribbon structure exists in the flat membrane and is not a product of membrane modification.

The tubular cristae reported here are not new. Similar but less extensive structures have been seen in a variety of mitochondria (for review, see Munn [20]), though the interpretation of just what is being seen has been uncertain. In the present study, the cristae have been rearranged completely to this morphology, which could be unequivocally identified as tubular from its appearance in thin section. From here, we conclude that the double row of TRU seen by negative staining does in fact represent two ribbons of TRU rather than a flat crista seen edge-on. This conclusion is independently supported by the occurrence of similar ribbons isolated from the membrane continuum in exchangease preparations. We are thus led to propose that ribbon structure is a property of the normal flat membrane, a proposal consistent with the appearance of the membrane by negative staining and freeze-fracture.

The technique of negative staining is often criticized because of the unknown effect of the staining and drying process on the structure observed [19, 21, 22]. The identification of tubes in thin section, as well as by negative staining, is therefore an important piece of evidence against their being artifacts of the negative staining process. However, glycerol pretreatment of samples for freeze-fracture was found to cause reversion of the tubes to a much wider diameter, as judged by their appearance in freeze-fracture specimens and in thin sections, presumably because the narrow tubes are osmotically sensitive. The tubular ETP was more stable; freeze-fracture revealed structures similar to those seen by negative staining, but rather more difficult to interpret (Fig. 1b, inset). Headpiece–stalks are also clearly visible in thin section (Fig. 2b, arrows). These may well be artifacts in the sense that we have employed conditions which render this structure highly visible [2], and yet the normal (if not enhanced) coupling properties of the tubular ETP and exchangease particles show that this is a viable conformation of the transducing unit. The degree to which headpieces are extended during energy transduction remains an open question.

The physical explanation of why such narrow tubular cristae are produced by the treatment described is at present obscure. We have found

the most significant factors in forming such tubes to be the use of frozen mitochondria and the hypotonic swelling step at room temperature. Fortunately, an understanding of this process (though desirable) is not necessary to an analysis of the resulting structure and its relationship to the normal membrane. We envisage the rearrangement of cristae to a microtubular morphology as occurring by invagination of a single ribbon to form a short tube; the process is then continued and promoted indefinitely by end-to-end association of further ribbons from the normal flat membrane. Since the ribbons in the tubular cristae are of indefinite (but enormous) length, it appears that short ribbons are capable of association to form longer ribbons. It may be meaningless to ask how long a normal ribbon is, since there may exist within the membrane a dynamic state of flux of ribbon lengths.

The tubular cristae studied here not only demonstrate the reality of ribbon structure, but also illustrate, *par excellence,* one functional capability of such structure, namely, the ability to modulate membrane shape. This is most dramatically demonstrated by the tubular ETP (Fig. 4a), where constraints applied by the rest of the mitochondrial structure have been removed. If the ribbon appearance were caused by some spurious alignment of noninteracting basepieces, these particles would have no backbone to maintain their highly asymmetric shape and would have rounded up on sonication. Some such backbone is needed to offset the energetically unfavorable high surface curvature of the lipid, and the two ribbons appear to provide it. It scarcely needs to be said that these particles are totally unexpected on the basis of the fluid mosaic model of membrane structure [3], and represent a vindication of the concept of a protein continuum [1]. Much interest is currently focused on the role of cytoplasmic filaments in controlling cell shape [23]; by showing extreme membrane shape changes in the absence of external filaments, this study indicates that intramembranous filaments or ribbons could equally well be involved.

The phenomenon of ribbon structure provides a rationale for several hitherto bizarre observations made on the mitochondrial inner membrane. Munn [20] has found that when the tubular cristae of tetrahymena are observed by negative staining, there is often only a single row of headpieces, alternating from one edge of a crista to the other. This appearance was interpreted as a single ribbon which spirals around the crista, but this interpretation only makes sense in terms of the concept of ribbon structure proposed here. The observation of a single spiral ribbon also eliminates the interpretation of ribbons as edge artifacts, since the edge opposite the rows of headpieces is bare.

A long-standing observation which ribbon continua explain nicely is the

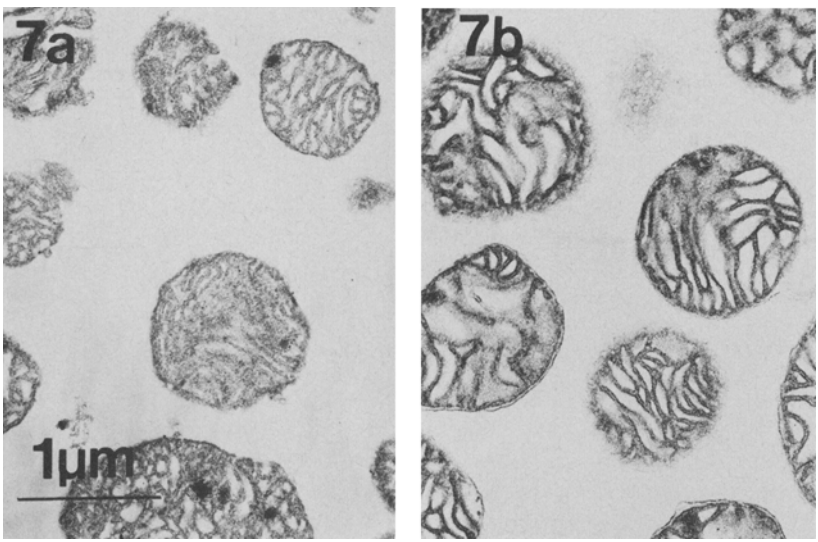

Figure 7. (a) Mitochondria 94% depleted of lipid; (b) heavy beef heart mitochondria. ×23,500.

appearance of mitochondria in thin section after lipid extraction [9]. At the time, the retention of the unit membrane profile was a source of amazement, since the prevalent view of membrane structure predicted that, in the absence of lipid, this profile should disappear. An understanding of the observation had to await the advent of the concept of intrinsic membrane proteins. However, there is a further puzzling aspect of the appearance of lipid-depleted mitochondria, and that is the extensive retention of the membrane continuum. In outline, the lipid-depleted mitochondria (Fig. 7a) appear strikingly similar to normal mitochondria (Fig. 7b). In terms of models of membrane structure in which lipid forms the only continuum [3], the membrane might be expected to fall apart on lipid extraction. However, if the protein is arranged in ribbons, a network of protein can hold the membrane together and maintain its original morphology.

Fragmentation of the mitochondrial inner membrane by procedures not involving the addition of lipid-disrupting agents also provides support for the concept of ribbon structure. Solubilization of the mitochondrial inner membrane by extended sonic oscillation yields, after centrifugation at $79{,}000 \times g$, a supernatant containing all the electron transfer complexes in particulate form [24]. By negative staining, particles measuring 80 to 100 Å in diameter are seen, frequently arranged in linear arrays.

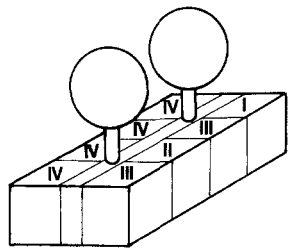

Figure 8. The proposed arrangement of electron transfer complexes with respect to the TRU ribbon.

Linear structures have also been observed by Crane et al. on fragmentation of mitochondria with detergents [25]. Of particular relevance are the fractions obtained with Triton X-114 treatment which show fibers with headpieces attached. Crane and co-workers found that these fibers were depleted in cytochromes, and concluded that in the intact membrane the electron transport components would be grouped around this backbone. Our findings support this general conclusion, though we would dispute the arrangement which they propose.

One might ask whether there is any reason to believe that the complexes have any particular arrangement with respect to the TRU. Some models of energy coupling require no direct interaction between exergonic and endergonic centers [26], and in any case, the fluid mosaic model of membrane structure would lead us to expect interaction by collision to allow sufficient opportunity for coupling in models [27,28] in which proximity of coupling centers is required. However, there are reasons for believing that the interaction is considerable. First, an exact 1 : 1 stoichiometry exists between F_1 (the headpiece) and complex III [29]. Other complexes, though less accurately determined, are thought to occur in stoichiometric amounts [30]. This would be a coincidence if the interaction is by collision, since no stoichiometry would be necessary; a much better explanation is that the electron transfer complexes and TRU form larger complexes in stoichiometric amounts [30]. Second, it has long been known that complex IV is readily separated from the other complexes I, II, and III [31]. If each complex were afloat in a sea of lipid, one might expect each to be easily separated. Third, Junge and DeVault have found evidence that complex IV is immobilized in the membrane, from its failure to lose dichroism by rotation after photolysis of the cytochrome a_3–carbon monoxide complex [32]. The only explanation of this result which allows rotation of the complex about an axis perpendicular to the plane of the membrane requires that the plane of the

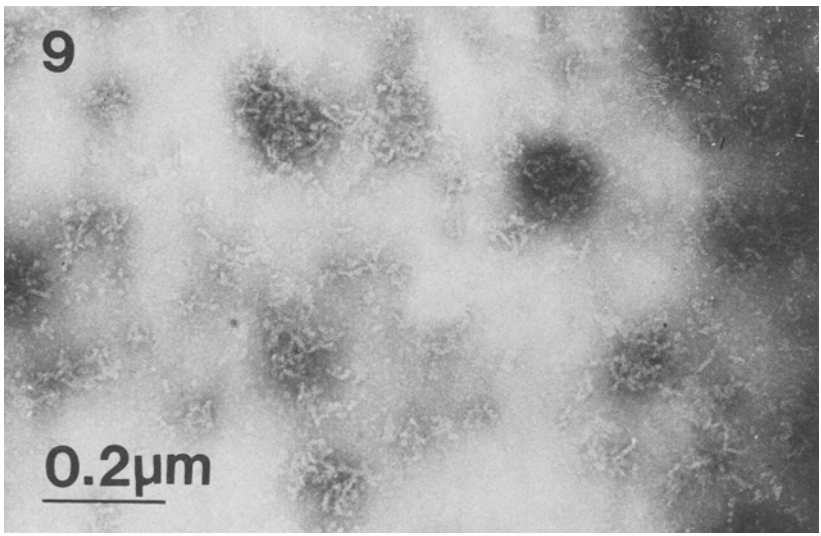

Figure 9. Linear aggregates of complex IV as seen by negative staining. ×85,000.

chromophore be oriented parallel to the plane of the membrane; there is as yet no independent evidence for this.

Knowing that the TRU form a ribbon is a great asset in attempting to deduce what the organization of the complexes with respect to the coupling center might be, since the number of possible arrangements which fulfil all requirements is greatly reduced. Indeed, one particular arrangement fits the data rather better than any other. This is shown in Fig. 8. Complex IV occupies one side of the ribbon, while complexes I, II, and III interact from the other side. Four observations lend support to this. First, this is the nicest rationale for the difference in extractability of the complexes. Crane et al. [25] have observed that the red fraction (containing complexes I, II, and III) prepared by the deoxycholate procedure of Hatefi et al. [33] contains fibers, indicating that complex IV has been split from the TRU ribbon plus the rest of the complexes. Further purification of complexes I, II, and III should be more difficult, as they are more intercalated. Second, the arrangement fits the observed stoichiometry of F_1 to complex III to complex IV [30]. The relative abundance of complexes I and II is less certain, but the classic experiments on reconstitution of the electron transport chain [34] favor the stoichiometry shown. Third, the dimensions of complex IV in the plane of the membrane have been found to be 87 ± 4 Å by 57.5 ± 2 Å from optical analysis of electron micrographs of crystalline arrays [35], and 68 Å in

diameter from freeze-fracture studies [7]. Thus, two complexes would well measure 115 ± 4 Å, a value in excellent agreement with the headpiece repeat distance [14]. Fourth, complex IV, as isolated by standard procedures, appears as linear arrays (Fig. 9; see also Chuang et al. [36]). Linear arrays are also visible in vesicles reconstituted from complex IV and soybean phospholipids when examined by freeze-fracture [37].

When the headpiece–stalk structures characteristic of the mitochondrial inner membrane were first observed by Fernandez-Moran et al. [14], they were observed to occur in rows lining membrane edges. Little has been made of this apparent linearity, because of the unknown effect of the negative staining process on membrane structure. The data presented here, however, give credence to their being a manifestation of the underlying organization of the TRU into ribbons. This has a far-reaching corollary: rows of headpiece–stalks have been observed in a wide variety of membranes, suggesting that ribbon structure is not unique to mitochondria.

Other evidence does exist for ribbon structure in nonmitochondrial membranes. Fragmentation of EDTA-treated chloroplasts and photosynthetic bacteria with Triton X-100 yields photoactive particles whose ribbon nature is dramatic [38]. A recent study of the appearance of various biological membranes after ultrastructural deformation led Wong and Geil [39] to conclude that each membrane contained protein fibers 100–300 Å in diameter and up to 4000 Å long. Membrane fracture during deformation caused the extrusion of fibers across the line fracture. The fibers were still seen after lipid extraction, but were susceptible to digestion with papain. The authors concluded that the observations did not agree with models of membrane structure which omitted extensive protein–protein interactions within the membrane; the results, however, are entirely consistent with the structure proposed here for the mitochondrial inner membrane.

Intercellular junctions are known to exhibit various modes of organization of intramembranous particles, as revealed by freeze-fracture [22]. The demonstration of linear aggregates in the mitochondrial inner membrane precludes the need to invoke forces external to the membrane for an understanding of the cohesion of these structures [3]; indeed, ribbon formation of the junction particles could well be a significant step in the genesis of intercellular junctions.

In conclusion, we have presented evidence for the organization of protein subunits of the mitochondrial inner membrane into ribbon continua which, by association, are able to modulate membrane shape. The view of the membrane which emerges from this is a marriage of concepts derived from models in which lipoprotein continua are dominant

[1,40] and models in which lipid forms the only continuum [3]. Here, the lipoprotein and the bilayer both form continua. The lipoprotein continuum consists of linear aggregates of tripartite units, of variable lengths, with electron transfer complexes grouped alongside, while the bilayer is interspersed between the lipoprotein ribbons. The bilayer lipid gives the membrane flexibility and fluidity [41], whereas the protein ribbons permit the organization necessary for coupling.

Note Added in Proof

Since this manuscript was submitted, it has been reported on the basis of e.p.r. spectra of oriented multilayers that the heme *a* plane of complex IV lies parallel to the plane of the mitochondrial membrane (J. S. Leigh and H. J. Harmon, *Biophys. J.*, **17** (1977) 251a). Thus, the only allowable conclusion from the data of Junge and Devault [32] is that complex IV in the normal membrane is immobilized.

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