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Review

The relevance of mitochondrial membrane topology to mitochondrial function

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Abstract

This review summarizes recent findings from electron tomography about the three-dimensional shape of mitochondrial membranes and its possible influence on a range of mitochondrial functions. The inner membrane invaginations called cristae are pleomorphic, typically connected by narrow tubular junctions of variable length to the inner boundary membrane. This design may restrict intra-mitochondrial diffusion of metabolites such as ADP, and of soluble proteins such as cytochrome *c*. Tomographic images of a variety of mitochondria suggest that inner membrane topology reflects a balance between membrane fusion and fission. Proteins that can affect cristae morphology include tBid, which triggers cytochrome *c* release in apoptosis, and the dynamin-like protein Mgm1, involved in inter-mitochondrial membrane fusion. In frozen-hydrated rat-liver mitochondria, the space between the inner and outer membranes contains 10–15 nm particles that may represent macromolecular complexes involved in activities that span the two membranes.
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Since the 1950s, electron microscopy (EM) has provided otherwise unattainable glimpses into the substructure of the cell. Among the more contentious issues in the early years of biological EM was the organization of the two membranes of the mitochondrion, the organelle that is the site of oxidative phosphorylation. These early controversies (reviewed in [1]) were resolved over the years as more sophisticated techniques became available for three-dimensional (3-D) imaging: serial section transmission EM (e.g., [2]), high-resolution scanning EM (e.g., [3]), electron tomography [4–9], and, most recently, cryo-electron tomography [6,10]. It is now firmly established that the invaginations of the mitochondrial inner membrane, called cristae, are not random wide folds in this membrane. Rather they are involutions of the inner membrane that can be extensive and topologically complicated, and that usually connect via narrow tubular segments to the inner boundary membrane, i.e., the region of the inner membrane that parallels the outer membrane on the mitochondrial periphery. The almost universal nature of this design principle for the mitochondrial inner membrane has raised several fundamental questions about its origin and possible functional significance.

This paper addresses several of the issues related to internal mitochondrial organization, with a focus on recent findings from electron tomography about inner membrane topology and its possible influence on mitochondrial function.

1. Topology implies a flexible and dynamic mitochondrial inner membrane

The shape of the mitochondrial inner membrane can vary tremendously among different organisms and tissues. However, the basic structures of most fungal, plant and animal mitochondria under normal physiological conditions are similar: a smooth outer membrane that envelops an inner

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membrane whose surface area is considerably larger and which envelopes a protein-rich matrix. To accommodate the volume constraint imposed by the outer membrane, the inner membrane has numerous invaginations, the cristae, each of which can have one or more tubular connections to the membrane periphery. This "typical" mitochondrial membrane morphology is exemplified in the tomographic reconstructions of isolated rat liver mitochondria in Fig. 1. The difference between the two reconstructions is that the mitochondrion of Fig. 1A was prepared by conventional chemical fixation, followed by osmication, dehydration, plastic embedding, sectioning and heavy-metal staining. The mitochondrion of Fig. 1B was simply deposited on an EM grid in a thin layer (approximately 500-nm thick) of sucrose-containing buffer, and plunge-frozen at liquid nitrogen temperature. It was in a frozen-hydrated state, embedded in vitreous (non-crystalline or amorphous) ice and imaged at low electron dose while maintained at near-liquid nitrogen temperature.

In order for the mitochondrial inner membrane to assume the variety of shapes observed, in situ and after isolation, it must be highly flexible. Moreover, the shape of the inner membrane can rapidly adjust in response to osmotic changes in the volume of the matrix. Matrix contraction results in expanded or dilated intracristal compartments, while matrix swelling collapses the intracristal space. When matrix expansion causes the surface area of the inner boundary membrane to exceed that of the outer membrane, the latter ruptures. (Note that this requires the cristal membrane regions to be recruited to the periphery, see below.) The pleomorphism and flexibility of the inner membrane in typical mitochondria is consistent with a membrane whose physical characteristics are determined by an underlying fluid phospholipid bilayer. This conflicts with the notion of "solid state", predominantly protein, cristae lacking extensive phospholipid bilayer [11]. However, there are numerous examples of mitochondria whose cristae appear regular and rigid, with angular or prismatic shapes (cf. [12]). The physical properties of the inner membranes in these "atypical" mitochondria may well be determined by some sort of protein infra- or superstructure.

The appearance of large cristae with multiple tubular connections to the peripheral inner boundary membrane in tomographic reconstructions of liver and neuronal mitochondria [5–8] suggests that they might form by merger or fusion of multiple tubular cristae. This is especially evident

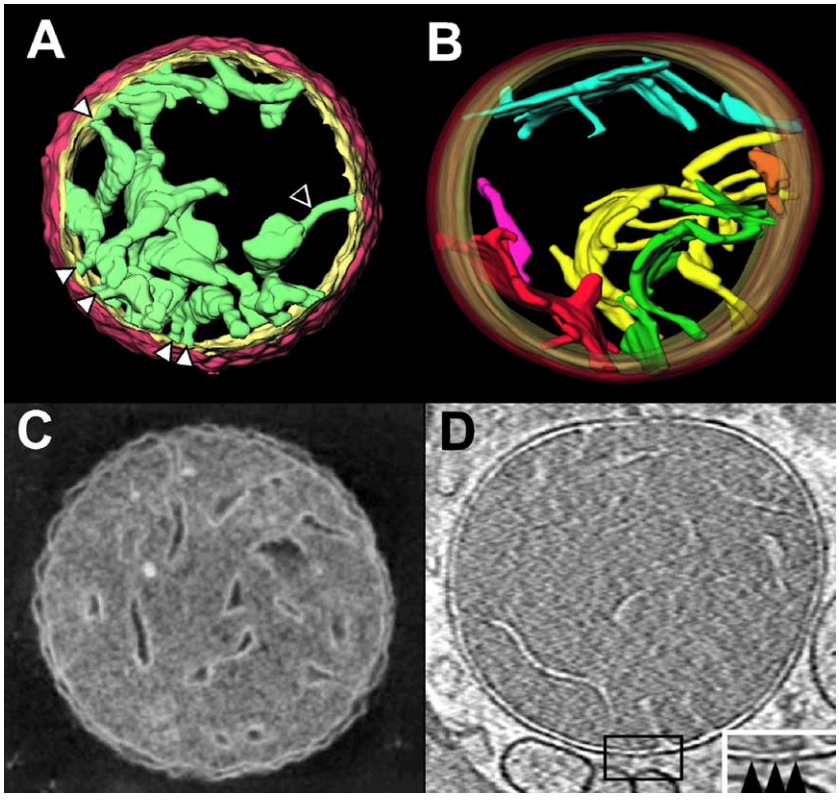

Fig. 1. Tomographic reconstructions of rat liver mitochondria. (A) Surface-rendering of the membranes in a condensed mitochondrion, prepared by conventional chemical fixation, plastic embedding and heavy-metal staining. Arrows point to tubular crista junctions. (B) Surface-rendering of the membranes in a mitochondrion suspended in 0.3 M sucrose, plunge-frozen at liquid nitrogen temperature and imaged in a cryo-electron microscope. (C, D) 2-nm-thick slices from the tomograms in A and B, respectively. Arrows in 2X inset of (D) point to 10–15 nm particles in intermembrane space. (A) is reproduced from [5] with permission of Elsevier Science. (B) and (D) are reproduced with modification from [6] with permission of IUBMB Life, Taylor Francis Group. In (A), (B) the outer membranes are red and the inner boundary membranes yellow. The cristae are green in (A) and multicolored in (B). The mitochondria in (A), (C) and (B), (D) are 1.5 and 0.7 μm in diameter, respectively.

in the tomogram of the frozen-hydrated liver mitochondrion in Fig. 1B. Several large cristae appear to be comprised of multiple, laterally aligned, tubular segments. Reversible swelling and contraction of mitochondria is a well-known phenomenon. For example, Hackenbrock showed that isolated rat liver mitochondria, during metabolic state changes, can reversibly switch between two inner membrane conformations: orthodox, corresponding to partial matrix expansion, and condensed, corresponding to partial matrix contraction [13]. Tomographic analysis indicates that the inner membranes in orthodox rat liver mitochondria are predominantly tubular with one or at most two connections to the periphery, while condensed mitochondria (as in Fig. 1) can have large cristae with three or more tubular junctions [4,6]. For the inner membrane to exhibit reversible transitions between these two topologies, it must undergo fusion and fission. In the cell, mitochondria are commonly seen to merge and divide, and the proteins that mediate the interactions of the four membranes (two inner and two outer) during these events are being characterized (e.g., [14,15]). It seems likely that one or more of the proteins involved in inter-mitochondrial membrane fusion and fission may have a role in intra-mitochondrial inner membrane fusion and fission as well (see below).

2. Characteristics of tubular inner-membrane junctions

The tubular connections of cristae to the inner boundary membrane were first termed *pediculi cristae* (the cristae's little feet) by Daems and Wisse [2] and later crista junctions by Perkins et al. [8], who determined that their circular diameters were fairly uniform in neuronal mitochondria (varying within a narrow range of 24–30 nm) [8]. However, just as crista morphology is generally not uniform, the junctions are not always short and circular [4,5,8]. For example, the crista junctions in the rat liver mitochondria of Fig. 1 can be either circular or somewhat elliptical, with dimensions in the range 20–40 nm. There is even more variability in the lengths of the membrane segments that connect the cristae to the inner boundary membranes. For example, while many of the crista junctions in the rat liver mitochondria of Fig. 1 are short (30–50 nm long), the tubular membrane segment indicated by the black arrowhead in Fig. 1A is 150 nm long. Mitochondria of the fungus *Neurospora crassa* appear to represent an extreme case, in which all the crista junctions are slot-like; openings with long dimensions up to 200 nm are observed, although the mean was 30–40 nm, similar to those in rat liver [10,16]. Despite this structural variability, the concept of the crista junction is a useful one, since it suggests a discrete, possibly specialized region of the inner membrane that, recent evidence suggests, may be highly conserved and functionally important.

Crista junctions are not rigid or unbreakable structures. For example, there are no indications that they prevent recruitment of inner membrane from the internal cristae to the periphery in response to large-scale matrix swelling. Rather, it has been shown that yeast mitochondria spontaneously form crista junctions after large-scale swelling (sufficient to totally unfold the inner membrane) and recontraction of the matrix [6,17]. Thus, the de novo formation of tubular junctions in the inner membrane is energetically favored. Renken et al. have suggested a thermodynamic model for formation of the crista junctions that explains their observed shape distribution, based on the theory of spontaneous curvature of lipid bilayers [18]. They conclude that the circular junction is a thermodynamically stable structure whose size and shape is influenced by relative matrix volume. Whether there are mitochondrial proteins involved in forming or stabilizing the tubular crista junctions is an open question. Such a role has been proposed for the protein mitofilin based on aberrant mitochondrial inner membrane morphology observed when its expression was down-regulated in HeLa cells [19].

The inner membrane of the relict mitochondrion in the anaerobic parasite *Cryptosporidium parvum* lacks crista junctions even though the membrane is highly convoluted [20]. This mitochondrion also lacks the normal respiratory chain complexes, although it might carry out other important physiological functions, such as non-heme iron metabolism. Keithly et al. proposed that the absence of tubular crista junctions might be an example of "reductive evolution", suggesting that the junctions are a bioenergetically important mitochondrial structure that might not be conserved in the absence of oxidative phosphorylation [20]. This, of course, raises the question: What functions might the tubular junctions serve in normal mitochondria?

3. Possible effects of crista morphology on mitochondrial function

3.1. Bioenergetics

Mannella et al. proposed that the narrow tubular junctions represent barriers to diffusion between the intracristal compartments and the intermembrane space ([5], also cf. [21]). They suggested, for example, that the junctions might prevent (or retard) dissipation of bulk-phase chemiosmotic H+ gradient from the intracristal space. Computer modeling, by Moraru and colleagues employing the Virtual Cell program [22], indicated that this is unlikely, given the expected buffering by weak acids like phosphate in the intracristal compartment [6]. However, full modeling of the influence of crista morphology on oxidative phosphorylation might be worthwhile, taking into account the possibility that protons pumped by the respiratory chain do not enter bulk phase but are confined to membrane surfaces, as well as the effect of extreme membrane curvature on local membrane electrical potential. The issue of restricted diffusion between mitochondrial compartments was next

examined using the Virtual Cell program for the case of ADP, a key metabolite whose cellular concentration is normally low (approximately 50 μM). Computer simulations indicated that, during normal steady state respiration, the concentration of ADP inside a large crista connected by a long narrow tubular segment to the intermembrane space (black arrowhead in Fig. 1A) drops to 20% of the external bulk concentration. This results in slower transport of ADP across the inner membrane (since intracristal [ADP] is now below the K_m of the adenine nucleotide transporter) and locally reduced efficiency of ATP production. Thus, the computer simulations predict that ATP generation can be regulated by inner membrane topology, by restricting diffusion of phosphate acceptor into the cristae. Interestingly, Hackenbrock showed that rat liver mitochondria display condensed conformation (large intracristal spaces, Fig. 1A) when ADP is in excess (the so-called respiratory state III) but revert to orthodox state (small intracristal spaces) when ADP is limiting (state IV) [13]. This change in crista morphology might serve to minimize the effects of restricted diffusion on ATP production when $[ADP]_{cytosol}$ is low. Whether this is the case, and whether there is a reciprocal energetic advantage associated with the condensed morphology when $[ADP]_{cytosol}$ is high, remains to be determined experimentally.

3.2. Apoptosis

Mitochondria occupy a central role in the cascade of events involved in programmed cell death (e.g., [23]). In particular, when certain members of the Bcl-2 protein family bind to mitochondria, they trigger release of pro-apoptotic proteins from the intermembrane/intracristal spaces. An example is cytochrome *c* which, when released from mitochondria, activates caspases (proteases) that are involved in cellular degradation reactions. Release of cytochrome *c* from mouse liver mitochondria is triggered by Bid which, in its truncated form tBid, induces oligomerization of Bax or Bak to form a large pore in the outer membrane [24]. In fact, a mitochondrial apoptosis-induced channel (MAC) in the outer membrane has been identified electrophysiologically and shown to be large enough to allow permeation of cytochrome *c* [25]. Scorrano et al. have shown that tBid has another effect on mouse liver mitochondria, namely it causes rapid "remodeling" of the inner membrane (Fig. 2A) [26]. Within a few minutes of

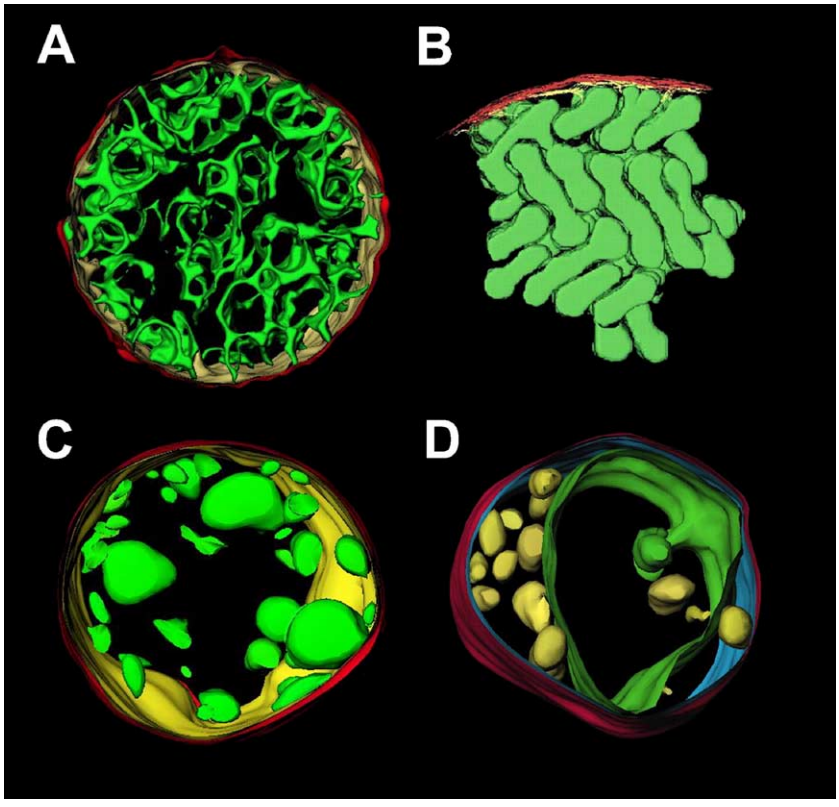

Fig. 2. Surface-rendered tomographic reconstructions of mitochondria having extreme inner membrane topologies. (A) Mouse liver mitochondrion after brief exposure to tBid [26]. (B) Amoeba mitochondrion during fasting. (Reproduced from [7] with permission of Elsevier Science.) (C) Yeast mitochondrion following extreme osmotic swelling and recontraction. (Reproduced from [6] with permission of IUBMB Life, Taylor Francis Group.) (D) Heart muscle mitochondrion from a patient with Senger's syndrome. (Reproduced with permission from M. Huizing, PhD thesis, Univ. of Nijmegen, 1998.) In (A–C) the outer membrane is red, inner boundary membrane yellow, and cristae green. In (D), the outer membrane is red, the vesicular cristae are yellow, the inner boundary membrane is blue and the second extended internal membrane is green. The mitochondria have diameters of (A) 0.9 μm, (C) 1.2 μm, and (D) 1.0 μm. The length of the outer membrane segment (red contours) in (B) is 0.8 μm.

exposure of mitochondria to tBid, the curvature of the inner membrane inverts and the size of the crista junctions increases. The matrix is confined to an intestine-shaped reticulum surrounded by an interconnected intracristal compartment. The dramatic change in inner membrane topology coincides with enhanced mobilization of internal cytochrome *c*, e.g., faster rates of NADH oxidation catalyzed by a cytochrome *c* redox shuttle between the outer membrane NADH dehydrogenase and the respiratory chain, and more complete release of cytochrome *c* following digitonin permeabilization of the outer membrane. These inner-membrane effects are independent of Bax/Bak-induced outer-membrane permeabilization. Scorrano et al. proposed that, in addition to its outer-membrane role, tBid might interact with one or more proteins in the inner membrane involved in regulating inner membrane topology. The implication is that normal inner membrane morphology might restrict cytochrome *c* diffusion out of the intracristal compartments, where the respiratory chain components tend to be concentrated [27]. Remodeling of the inner membrane by tBid apparently allows easier diffusion of cytochrome *c* into the intermembrane compartment by enlarging the intracristal compartment into a single interconnected space and increasing the openings of the cristae into the boundary membrane. Reed and Green caution against over generalizing this finding, since there are likely to be multiple mechanisms of cytochrome *c* release and the membrane remodeling reported by Scorrano et al. for liver mitochondria may not be universal [28]. For example, mitochondria with "remodeled" inner membranes have been observed following induction of apoptosis in mouse fibroblasts [26] but not in Jurkat cells (C.W. Renken, PhD Thesis, San Diego State University, 2004). Nonetheless, efforts are underway to identify possible inner-membrane partners for tBid. One candidate is the adenine nucleotide transporter (ANT), since inhibitors have been shown by Klingenberg and co-workers to induce a dramatic change in inner membrane topology similar to that induced by tBid [29]. Since ANT comprises a significant fraction of the total protein mass in the mitochondrial inner membrane, it would not be surprising if an inhibitor-induced conformational change in this protein altered membrane curvature.

3.3. Mitochondrial biogenesis

Perkins et al. have suggested a possible role for crista junctions in segregating respiratory complexes between the inner boundary membrane and cristae [8]. Recent immunolabelling experiments by Gilkerson et al. tend to support this hypothesis [27]. Their data indicate that two inner membrane complexes (complex III and ATP synthase) are concentrated over 2-fold in the cristae. It is possible that the large respiratory complexes are assembled on the inner boundary membrane (proximal to the outer membrane protein import machinery), and that cristae form by movement of the assembled complexes and lipid through the crista junctions [16]. This continuous inward flow of inner membrane components through narrow tubular junctions might inhibit back diffusion of the respiratory complexes, resulting in their observed concentration in the cristae. If this was, indeed, the mechanism of cristae formation, one might expect the respiratory complexes to equilibrate between cristae and boundary membrane following osmotic swelling and recontraction of isolated mitochondria.

4. Atypical inner membrane topologies

4.1. Balance between inner membrane fusion and fission

The above discussion, for the most part, is centered on a definition of "typical" mitochondria exemplified by the rat liver organelle, which has pleomorphic inner membrane compartments that connect to the boundary membrane and to each other by narrow tubular junctions. In fact, the variety of inner membrane morphologies displayed by mitochondria across different species, different cell types within the same species, and different metabolic states within the same cell is almost endless. See the monograph by Munn for examples [12]. The observed inner membrane morphologies can be characterized or categorized in many ways. One is according to the interconnectedness of cristae, for which two obvious extremes can be defined: inner membrane topologies in which the cristae are interconnected to form a continuous internal compartment, and topologies in which the inner membrane is fragmented to form multiple discrete compartments. An example of a high degree of interconnectivity is the topology observed following exposure of mouse liver mitochondria to tBid (Fig. 2A) [26]. Another is found in mitochondria of the amoeba *Chaos carolinensis* following onset of fasting. Within 24 h of food withdrawal, the cristae switch from tubular morphology, common in protozoan mitochondria, to a paracrystalline network of interconnected compartments with cubic symmetry (Fig. 2B) [30]. The latter structural transition might be functionally relevant, e.g., it may be part of a protective response mechanism that helps the mitochondria deal with increased rates of reactive oxygen generation during fasting [31]. The opposite extreme in inner membrane topology, detached or vesicular cristae, is observed in some yeast mitochondria following extreme osmotic swelling and recontraction (Fig. 2C) [6,17] and in muscle mitochondria from patients with a particular myopathy, Senger's syndrome (Fig. 2D) (M. Huizing, PhD Thesis, University of Nijmegen, 1998). Interestingly, the molecular defect in Senger's syndrome has been tentatively characterized as a deficiency in ANT (M. Huizing, PhD Thesis, Univ. Nijmegen, 1998). As noted above, the "typical" morphology displayed by the inner membranes of rat liver and neuronal mitochondria has been attributed to inner membrane fusion and fission. We propose further that extreme inner membrane topologies like those in the mitochondria of Fig. 2 might represent situations in

which one or the other process, fusion (Fig. 2A, B) or fission (Fig. 2C, D) is dominant. What remain to be determined are the identities of the proteins that influence, directly or indirectly, the intra-mitochondrial fusion and fission of the inner membrane.

4.2. Proteins that affect inner membrane morphology

In the past few years, several proteins have been implicated in maintaining normal inner membrane morphology. In at least one case, the protein is also known to be involved in inter-mitochondrial membrane fusion, the dynamin-like protein Mgm1 [32,33]. Mitochondria in cells in which Mgm1 is defective or down-regulated have been described as having aberrant cristae [32,34]. The nature of the structural deficiency is currently being characterized by electron tomography in nematode and yeast model systems (van der Bliek, A., Pain, D., Mannella, C.A., unpublished data). Defects in Mgm1 have been identified as the molecular basis of Dominant Optic Atrophy in humans, underlying the physiological importance of mitochondrial dynamics and, perhaps, maintenance of proper inner membrane morphology. Another modulator of cristae morphology is ATP synthase, which normally is present in an oligomeric state in the inner membrane [35]. Oligomerization is blocked in yeast cells deficient in either the e or g subunits of ATP synthase, and the mitochondria have aberrant, onion-like cristae [35]. Pain and co-workers recently have reported that Mgm1 regulates the stability of subunit e, also called Tim11 [34]. This raises the possibility that the effect of Mgm1 defects on mitochondrial inner membrane morphology might be due, at least in part, to failure of ATP synthase to oligomerize.

5. Nature of the contacts between the mitochondrial outer and inner membranes

The existence of physical contacts between the mitochondrial outer and inner membranes was proposed by Hackenbrock based on transmission electron micrographs of conventionally fixed and plastic embedded suspensions of mitochondria [13]. A slice from the tomogram of a conventionally prepared rat-liver mitochondrion (Fig. 1C) shows numerous sites of apparent contact between the inner and outer membranes along the periphery of the organelle. Inter-membrane contacts have been implicated in several important mitochondrial processes, including protein import and metabolite channeling (e.g. [36–38]). However, tomographic reconstructions of unfixed, unstained, frozen-hydrated *Neurospora crassa* [10] and rat liver mitochondria [6] have indicated that there are far fewer sites where the mitochondrial outer and inner boundary membranes make contact (pinch together) than inferred from images of chemically fixed and dehydrated specimens—on the order of 1 to 10 per mitochondrion by extrapolation from the few cases observed ([10] and Mannella, unpublished data). Instead, intact, isolated, frozen-hydrated rat-liver mitochondria contain numerous 10–15 nm particles in the space between the outer and inner boundary membranes, which keep a constant separation (inset, Fig. 1D) [6]. The presence of "bridging particles" in the inter-membrane space also has been reported by Senda and Yoshinaga-Hirabayashi based on quick-freeze, deep-etch EM [39] and by Perkins et al. using electron tomography [16]. These particles might represent membrane-spanning protein complexes involved in one or more of the activities expected to occur at contact sites and may, in fact, physically link the outer and inner membranes together, either permanently or transiently.

The discrepancy in appearance between frozen-hydrated mitochondria (smooth membranes with constant separation along the periphery; Fig. 1B, D) and conventionally prepared mitochondria (puckered membranes with numerous apparent sites of contact: Fig. 1A, C) likely has a simple explanation. The latter membranes are wrinkled, probably due to one or more of the processing steps (e.g., chemical fixation, organic solvent dehydration), resulting in numerous spurious or incidental contacts between the outer and inner boundary membranes. Some of the apparent contacts, in fact, might be sites of physical attachment of the membranes (possibly occurring at the 10–15 nm particles), but any real contacts would be difficult to distinguish from random membrane puckering. It is worth noting that the appearance of the frozen-hydrated mitochondria (smooth membranes, relatively few contacts) is similar to that of cross-fractured, unfixed rat liver mitochondria obtained by freeze-fracture electron microscopy (e.g., [40]) and of tomographic images of mitochondria in frozen-hydrated sections of rat liver ([41] and Hsieh, C., Marko, M., Mannella, C.A., unpublished data). Whether some or all of the 10–15 nm inter-membrane space particles physically attach the two membranes together cannot be determined from tomograms of intact organelles. However, there are numerous other indications for physical attachment of the two mitochondrial membranes, particularly the difficulty encountered separating outer from inner membranes following mitochondrial lysis (e.g., [42]).

6. Conclusions and future directions

Of all cellular organelles, the mitochondrion has been the most intensely studied by electron microscopy (a fact easily confirmed by a literature database search). The reasons for this are obvious, chief among them the central importance of this organelle to cellular physiology, coupled with its structural and functional complexity. This review has focused on a dimension to mitochondrial research that has been largely overlooked since the 1960s, when the old canonical model for mitochondrial structure first took hold. The question is whether the fundamental design of the mitochondrial inner membrane and the changes in shape

caused by metabolic state transitions, apoptosis inducers, and other effectors are meaningful or mere epiphenomena. We posit that the former is true, that ultimate understanding of processes like oxidative phosphorylation and apoptosis will inevitably take into account their structural context, including the influence of mitochondrial membrane topology on internal diffusion and compartmentation. Likewise, identifying the factors that control mitochondrial membrane shape and dynamics and, in turn, understanding how these factors are regulated by the cell should provide collateral evidence for significance. Increasingly sophisticated tools are needed for probing and modeling mitochondrial function, as well as for imaging the organelles under conditions that are as close to native as possible. Important progress is being made toward the goal of high-resolution 3-D electron imaging of cellular substructure in frozen-hydrated cells [43] and tissue [41]. It is hoped that continued improvements in the technique of cryo-electron tomography will open wide a new window on the study of cellular bioenergetics.

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