Abstract

In recent years, electron tomography has provided detailed three-dimensional models of mitochondria that have redefined our concept of mitochondrial structure. The models reveal an inner membrane consisting of two components, the inner boundary membrane (IBM) closely apposed to the outer membrane and the cristae membrane that projects into the matrix compartment. These two components are connected by tubular structures of relatively uniform size called crista junctions. The distribution of crista junction sizes and shapes is predicted by a thermodynamic model based upon the energy of membrane bending, but proteins likely also play a role in determining the conformation of the inner membrane. Results of structural studies of mitochondria during apoptosis demonstrate that cytochrome \(c\) is released without detectable disruption of the outer membrane or extensive swelling of the mitochondrial matrix, suggesting the formation of an outer membrane pore large enough to allow passage of holo-cytochrome \(c\). The possible compartmentation of inner membrane function between the IBM and the cristae membrane is also discussed.

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1. Introduction

Mitochondria were among the first subcellular organelles examined by electron microscopy. In the early 1950s, both Sjostrand and Palade observed that mitochondria contained more than one membrane but differed initially in their models of the three-dimensional structure. Sjostrand’s [1] early model identified the outer and inner boundary membranes (IBM) but contained a third membrane forming septa dividing the matrix into multiple compartments. Palade [2] also identified outer and IBM, but in his model, the inner membrane curved inward to form baffles that he called crista mitochondriales. The model currently depicted in textbooks is essentially that of Palade with the mitochondrial boundary defined by the smooth outer membrane containing an inner membrane with a much larger continuous closed surface accommodated within the space defined by the outer membrane through the formation of broad folds forming cristae. In this “baffle” model, the outer membrane and the inner membrane define two internal compartments, the intermembrane space between the two membranes and the matrix within the inner membrane. The intracristal space within the cristae folds is continuous with the intermembrane space communicating through broad openings and was not generally considered to comprise a separate compartment. Until recently, this has been the generally accepted model of mitochondrial structure [3,4].

As the details of mitochondrial respiration were elucidated, several laboratories investigated structural changes in isolated mitochondria in response to respiration rates controlled by metabolic substrates, oxygen, and ADP. Hackenbrock’s studies were the most detailed as he examined the changes in the structure of isolated mitochondria in States I–V previously defined by Chance and Williams [5]. Mitochondria observed by electron microscopy in situ have a relatively large matrix volume that pushes part of the inner membrane up against the outer membrane with a small space between them; we call this the IBM. The remaining inner membrane forms cristae projecting into the matrix, and the opposing membranes of these cristae are close together with a small space between them. Hackenbrock called this conformation “orthodox”. When mitochondria...
are isolated, the matrix normally contracts owing to osmolarity effects causing the intermembrane space and the intracristal spaces to swell pulling the IBM away from the outer membrane except at contact sites where the two appear to be connected (although not fused); Hackenbrock called this conformation “condensed”. Hackenbrock observed that isolated State III mitochondria characterized by high respiration rates generated by high substrate, ADP, and oxygen concentrations have a condensed conformation that has long been associated with rapidly respiring mitochondria. Conversely, isolated State IV mitochondria that respire more slowly owing to low concentrations of the ADP phosphate acceptor have a relatively large matrix with the IBM membrane closely apposed to the outer membrane characteristic of the orthodox conformation [6]. It is tempting to associate actively respiring mitochondria in situ with the condensed conformation observed with isolated mitochondria, but mitochondria observed in situ are almost always in the orthodox conformation; thus, the relevance of the condensed morphology in living cells is unclear.

2. Electron tomography and the crista junction paradigm of mitochondrial structure

Although electron microscopy of conventionally prepared thin sections of cells and tissues has provided a wealth of information on the ultrastructure of mitochondria, deducing the three-dimensional structure of a complex object from individual thin slices can be perilous. Three-dimensional reconstruction of the mitochondria in yeast cells from serial thin sections has shown that in many cases, the mitochondria form a complex interconnected reticulum rather than compact individual mitochondria that are assumed from the profiles observed in single thin sections [7]. Interconnected mitochondrial networks have also been observed in other cell types such as Euglena, and HeLa cells [8,9], while in other cells, individual mitochondria are elongated, probably through interactions with the cytoskeleton. The interpretation of inner membrane topology from thin section electron micrographs is likewise prone to error. Indeed, Daems and Wisse [10] examined serial thin sections of mitochondria and noted that the connections between cristae and IBM were not observed as frequently as one would expect if the baffle model is correct. They proposed that these connections are 30-nm-diameter tubular structures, but this model was not widely accepted.

Electron microscopy of semithick sections, ca. 0.25–0.5 µm, requires an electron microscope operating at significantly higher accelerating voltage in order to produce an electron beam that will penetrate the specimen without significant loss of energy that blurs the image due to inelastically scattered electrons. High voltage electron microscopes (HVEM) operating at 1000 kV have been available for many years but only at a few facilities. National resources operating intermediate voltage electron microscopes (IVEM at 400 kV) have increased access and interest in electron microscopy of semithick specimens. The ability to form an image of a semithick specimen, however, is only the first step in understanding its detailed structure, because so much of the structure overlaps when projected onto the two-dimensional image plane. Examining stereo images improves the situation somewhat but is an inadequate solution to the problem, because it is difficult to follow accurately the membrane topology throughout the entire volume. Medical X-ray imaging faced a similar situation that was solved by computerized axial tomography (CAT scans) in which many X-ray images are recorded at closely spaced angular intervals and recombined by computer to generate a three-dimensional X-ray image. The same principle applies to electron microscopy; one can tile the specimen at small regular intervals recording a tilt series, typically 60–120 images at 2–1° intervals from $-60°$ to $+60°$. After scaling and aligning the images to a common origin, they can be used to calculate a three-dimensional electron micrograph, an electron tomogram, using the same computer algorithms employed in medical imaging, most commonly the R-weighted filtered backprojection [11].

Electron tomograms contain a great deal of information (Fig. 1b). Although much of this information can be gleaned by looking at individual slices of the tomogram, understanding the structure as a whole often requires creation of a three-dimensional model based upon segmentation of the features of interest. In the case of electron tomograms of cellular structures such as mitochondria, this usually involves tracing membrane profiles in each of many parallel sections of the tomogram to form a stack of membrane contours. The stack of contours is interpreted by a display program as a three-dimensional surface that can be rotated and viewed at any angle [12]. Fig. 1c views the model constructed from the tomogram of the mitochondrion in Fig. 1a, while Fig. 1d shows a model of the same mitochondrion containing only four separately segmented cristae [13]. The tilt series, tomogram, and three-dimensional models rotating about an axis can be viewed in the form of Quicktime movies at the website specified by the following URL: http://www.sci.sdsu.edu/TFrey/MitoMovie.htm.

Viewing the tilt series as a movie shows that it contains a great deal of structural information with views along some directions containing circular profiles not predicted by the baffle model (Fig. 1a, arrows). Successive slices of the reconstruction show that the connections between cristae and the IBM are limited in extent (Fig. 1b, vertical arrows), the same observation made by Daems and Wisse [10] in serial thin sections. Resectioning the tomogram in a plane parallel to the optic axis ($z$-axis) and perpendicular to a crista at the region where it connects to the IBM shows connections that can be approximated as a tube 28 nm in diameter (Fig. 1b, horizontal arrows). Similar results have been obtained by Mannella et al. [14–16]. We have called these tubular connections between cristae and IBM crista junctions (CJs). From our measurements of the diameters of
CJs in mitochondria from rat, mouse, and chick neural tissue, rat brown adipose tissue, Jurkat cells, *Xenopus* egg cells, and *Neurospora crassa*, we have found that the distribution of diameters is, within experimental error, the same for all, 28 ± 6 nm [13,17–20]. This includes mitochondria in *Neurospora* during inhibition of protein import through the TOM 19 complex in the outer membrane. In this study, cristal membrane area decreased within the first 8 h following inhibition of protein import, while mitochondrial volume appeared unchanged. After 16 h of inhibition, mitochondrial volume decreased with further loss of cristal membrane, and after 32 h, mitochondria were markedly smaller and cristae nearly disappeared. Throughout the experiment, however, cristae maintained lamellar structures with multiple CJs connecting them to the IBM. However, the cristae became smaller, fewer in number, and the number of CJs decreased in proportion to the crista volume [18].

Other structures that we have measured in electron tomograms are also equivalent among all tissue and species; these include the distance from the outer surface of the outer...
membrane to the inner surface of the IBM (OM–IM), the sizes of contact sites, and the width of the crista. The only significant differences we have found were in Neurospora mitochondria that contain a population of CJs that were somewhat elongated or elliptical in cross-section, while others were circular in cross-section with the same 28-nm diameter [18]. Elliptical or slot-like CJs were also observed in isolated Neurospora mitochondria observed frozen in thin layers of ice [21]; however, the axial ratios of these elliptical CJs were much larger than those found in situ in chemically fixed cells and may have been affected by compression during freezing in thin layers of ice. Chemically fixed isolated mitochondria from rat liver and from Xenopus also have large elliptical junctions [29]. However, these types of junctions may result from the isolation procedure, which often causes the matrix to contract. We have also examined mitochondria in brown adipose tissue prepared by cryofixation (ultrarapid freezing of live tissue) followed by freeze substitution comparing them with tissue prepared by conventional chemical fixation/resin embedding. When performed in an optimal way, cryofixation followed by freeze substitution is believed to provide better structural preservation and serves as a good test of our observations in chemically fixed tissues. Measurements of CJ diameters, contact sites, and cristae widths are identical for both chemically fixed and cryofixed specimens, but the OM–IM measurement is 5 nm smaller in cryofixed specimens (17 nm) than in chemically fixed specimens (22 nm). This might indicate that conventional chemical fixation causes a slight contraction of the matrix pulling the IBM a few nanometers from the outer membrane. Alternatively, the smaller separation between OM and IBM could be an artifact of freezing that in other poorly frozen areas of the specimen causes collapse of the crista membranes [17].

3. What controls the shape of crista junctions?

Two observations have directed our research efforts into the forces that control the shape of crista junctions. First, in a study of Neurospora mitochondria in which protein import through the outer membrane was inhibited, crista surface area decreased linearly with time. CJ numbers decreased at the same rate, indicating that CJs are not permanent structures [18]. If they are formed by some kind of protein architecture, it must be able to disassemble when fewer junctions are needed. Alternatively, cristae junctions may be

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**Fig. 2.** (a) The cristae in the model of Fig. 1c viewed from the back with CJs outlined in red. (b) An idealized model of a CJ defined by two principal radii of curvature, $R$ the size of the CJ and $r$ the flare of the CJ. (c) The distribution of CJ sizes, $R$ values, measured from different tomograms. The distribution fits an exponential Boltzmann-like distribution significantly better than a normal distribution as determined by calculation of chi-squared values for the best fit in each case (adapted from Renken et al. [20]).
an energetically stable membrane structure that forms and disappears spontaneously responding to changes in inner membrane surface area and matrix volume. This is consistent with the observation of Mannella et al. [22] that CJs form spontaneously following rapid swelling and partial contraction of purified yeast mitochondria. Second, we found that an exponential Boltzmann distribution fits the observed distribution of crista junction diameters with a chi-squared value approximately one half that for a normal distribution (Fig. 2c; here, CJ size is expressed as the radius from the center of the CJ to the center of the bilayer; by this definition, the average crista junction radius is 10.5 nm). This suggests that the distribution of crista junction size is a thermal fluctuation about a shape and size of minimal energy.

We have created a thermodynamic model based upon established theories for the energetics of membrane bending developed by Helfrich [23] and others by which the energy of membrane bending for a surface of constant topology is defined by

\[ E_b = \frac{K_b}{2} \oint (C_1 + C_2 - C_0)^2 \, dA + G \]

Here, \( G \) is a constant and \( K_b \) is the bending modulus for the membrane, essentially a measure of how rigid the membrane is or how much energy it takes to bend it. \( C_1 \) and \( C_2 \) are the two perpendicular principal curvatures that define the shape of the bend; they are the inverse of the radii of curvature and therefore have units of nm\(^{-1}\). \( C_0 \) is the spontaneous curvature of the membrane, the tendency of the membrane to curve without an imposed force, and is equal to zero for a bilayer symmetric in lipid composition and other environmental conditions (pH, ionic strength, etc.). In the case of the mitochondrial inner membrane, however, the pH differs by approximately one unit on opposite sides of the membrane. Furthermore, the lipid composition on opposite bilayer leaflets at a crista junction could be different giving the bilayer a nonzero spontaneous curvature, \( C_0 \). We have derived an expression for the energy required to bend the inner membrane at a model crista junction defined by two parameters, \( R \) and \( r \) shown in Fig. 2b. \( R \) and \( r \) can be measured from tomograms and are the two principal radii of curvature describing what we will call the size (\( R \)) and flare (\( r \)) of the crista junction; they determine \( C_1 \) and \( C_2 \), respectively. In fitting our data to the thermodynamic model, we obtained values of \( K_b \) of approximately 3 kT and \( C_0 \) of approximately 0.01 nm\(^{-1}\). Membranes typically have values of \( K_b \) in the range of 10 kT, so the mitochondrial inner membrane in the region of crista junctions appears to be more easily bent. This may reflect the fact that we allowed \( C_0 \) to assume optimal nonzero values [20]. The relatively small \( K_b \) suggests that crista junctions may be readily deformed accounting for the elliptical crista junctions found in \( N. \) crassa mitochondria in situ [18], in tomograms calculated for isolated frozen-hydrated mitochondria [21,22], and in the Class II remodeled mitochondria described by Scorrano et al. [24].

4. Mitochondria in apoptosis

In most, but certainly not all pathways of apoptosis (programmed cell death), mitochondria play an early role by the release of cytochrome \( c \) from the intermembrane space and perhaps the intracristal space in response to one or more of a variety of signals including presence of oxidants, ceramide, high [Ca\(^{2+} \)], or proapoptotic proteins such as Bax. Cytochrome \( c \) triggers apoptosis as a component of the apoptosome that activates caspase 9 that subsequently activates other downstream caspases. If caspase activation is inhibited, loss of cytochrome \( c \) can lead to slower necrotic cell death as the loss of cytochrome \( c \) from mitochondria inhibits electron transport and ATP production [25]. The structural question that we have addressed by electron microscopy/tomography is "How is cytochrome \( c \) released?" Release of cytochrome \( c \) from the intermembrane space requires openings in the outer membrane large enough to allow the approximately 3-nm diameter holocytochrome \( c \) to pass. Two mechanisms have been proposed to effect cytochrome \( c \) release. The first is based upon swelling of the mitochondrial matrix to an extent that its outward pressure ruptures the outer membrane releasing all of the contents of the intermembrane space. This mechanism provides a role for a long-studied phenomena observed in isolated mitochondria, the permeability transition (PT) [26]. Certain stimuli such as high [Ca\(^{2+} \)] and or oxidants can cause the opening of a high conductance channel in the inner membrane of isolated mitochondria resulting in the loss of the electrochemical proton gradient, and the hyperosmolarity of the matrix causes it to expand to the point that it can rupture the outer membrane. Support for this model lies in the observations that some inducers of the PT can cause outer membrane rupture and apoptosis and inhibitors of the PT have been reported to also inhibit apoptosis in some systems [25]. However, the observation that cytochrome \( c \) can be released before loss of the inner mitochondrial membrane potential suggests another mechanism, the formation of an outer membrane pore large enough to allow holocytochrome \( c \) to pass into the cytosol. Pavlov et al. [27] have reported conductance measurements on the mitochondrial outer membrane that are consistent with the formation of a 4-nm diameter pore that correlates with the onset of apoptosis.

We examined the structures of apoptotic mitochondria in collaboration with Drs. Don Newmeyer and Doug Green at the La Jolla Institute for Allergy and Immunology. In the \textit{Xenopus} cell-free system, purified mitochondria release cytochrome \( c \), inducing the downstream events of apoptosis. Release of cytochrome \( c \) requires the presence of cytosol or proapoptotic proteins Bax or Bid and is inhibited by Bel-2, an anti-apoptotic protein. Examination of \textit{Xenopus} mito-
chondria by electron microscopy and by electron tomography showed that the mitochondria, both apoptotic and nonapoptotic, were not swollen and were in fact very condensed pulling the IBM away from the outer membrane, swelling the intracristal spaces, and expanding the sizes of cristae junctions to the point that they are difficult to define. This matrix condensation is probably the result of the isolation procedure as is commonly observed with purified mitochondria [28]. Electron tomograms of both control and apoptotic Xenopus mitochondria show an intact outer membrane with no ruptures detectable at the resolution of the tomograms (estimated to be 7–10 nm) despite the loss of virtually all cytochrome c from the apoptotic mitochondria (Fig. 3). These results are consistent with the observation that these mitochondria do not appear to lose their membrane potential during release of cytochrome c, and continue to be able to import protein, a process that requires a membrane potential and ATP [29]. Swelling of the Xenopus mitochondrial matrix can be caused by inducing a PT with high [Ca\(^{+}\)], and these mitochondria often exhibit rupture of the outer membrane with release of cytochrome c (Fig. 3c). The very large openings observed between the intracristal and intermembrane compartments of the condensed Xenopus mitochondria may facilitate release of intracristal cytochrome c as suggested by Scorrano et al. [24] who observed an increase in CJ diameter in purified mouse liver mitochondria that have released cytochrome c in response to the pro-apoptotic protein BID.

We have subsequently studied mitochondrial ultrastructure in a Jurkat cell model of apoptosis induced with etoposide. Electron tomography is labor intensive and allows limited sampling of mitochondria, so in this case, we developed statistical methods to analyze a large number of mitochondrial membrane profiles in numerous cells sampled by thin section electron microscopy. Although initial inspection of the micrographs suggested that the mitochondria in apoptotic cells were swollen, careful measurements showed that they were not and only appeared swollen because apoptotic cells were smaller than non-apoptotic cells. Analysis of mitochondrial ultrastructure characterized by cross-sectional area, the perimeter of the outer membrane, and the perimeter of the inner membrane showed only subtle differences between normal and apoptotic mitochondria that might indicate a loss of protein import in apoptotic mitochondria. Although it may be possible to find individual examples of swollen mitochondria in either normal or apoptotic cells, the populations of mitochondria in these two cell types are nearly identical and neither indicate matrix swelling [30]. Our conclusions from our studies of the Xenopus cell-free model and the Jurkat/etoposide model of apoptosis is that cytochrome c release is not accompanied by mitochondrial swelling and outer membrane rupture, but cytochrome c is released through a large pore in the outer membrane. This does not mean that mitochondria do not swell in other instances of apoptosis or that the PT plays no role in apoptosis. Mitochondrial swelling with or without a PT may be an effect of apoptosis, or a PT may occur in the Jurkat/etoposide model without causing mitochondrial swelling. Mitochondrial swelling could be a cause of apoptosis in other systems, since rupture of the outer membrane would certainly release cytochrome c into the cytosol.

5. Future directions

These observations culminate in the crista junction paradigm of mitochondria structure in which the outer membrane limits mitochondrial volume in combination with the overall shape of mitochondria that may also be regulated by interactions with the cytoskeleton and unidentified mitochondrial proteins. Mitochondria in situ are normally in the orthodox conformation with a large matrix volume pushing the IBM against the outer membrane with a small space, the
intermembrane space, between them. The remaining inner membrane surface projects into the matrix forming cristae that connect to the IBM at circular crista junctions whose diameters are distributed over a relatively narrow range around an average of 28 nm. This structure is probably dynamic with CJs forming and disappearing in response to changes in environment, mitochondrial shape, inner membrane surface area, etc. Interestingly, the distribution of CJ diameters for swollen mitochondria (Fig. 3c) is 4 nm smaller than for normal orthodox mitochondria as would be predicted by a thermodynamic control of CJ diameter responding to increased matrix pressure. The relevance of the condensed conformation that is rarely observed in vivo is unclear. As the matrix condenses, CJs increase in diameter to the point that they are no longer discrete structures, and the IBM is pulled away from the outer membrane except at contact sites where protein transport from the cytosol into the matrix is believed to occur [31]. This could disrupt other transport complexes formed between IBM and outer membrane proteins.

Much remains to be learned about the detailed structure of mitochondria and how this affects function. While we have developed a credible thermodynamic model that predicts the conformation of CJs, there are a number of proteins that appear to control the conformation, fusion, and fission of mitochondrial membranes [32,33], and there are probably more that remain to be identified. Electron tomography can play an important part in characterizing the roles these proteins play by identifying their effects on membrane conformation and their sites of action within mitochondria. The discovery that the circular CJs that connect the IBM to cristae are much smaller than the broad folds described in the baffle model raises the question of whether CJs play a role in mitochondrial function. The size of CJs might constitute a barrier to diffusion of molecules such as ATP or ADP from the intracristal compartments to the intermembrane space and cytosol in actively respiring mitochondria, and simulations give credence to this possibility [22]. CJs might also inhibit full release of cytochrome c during apoptosis [24]. However, another role for CJs in the function of mitochondria could be to separate two inner membrane compartments with differing membrane protein compositions. In orthodox mitochondria, an efficient division of inner membrane function might result if the IBM were enriched with ion and metabolite transport proteins facilitating their movement between the cytosol and the matrix through associations between mitochondrial outer membrane porin and inner membrane transport proteins. The electron transport proteins, on the other hand, have no particular need to communicate directly with the cytosol and would function efficiently within the cristae membrane. Such compartmentation might be controlled by as yet unidentified proteins or could result passively if the ion and metabolite proteins were drawn to the IBM by interactions with outer membrane proteins forming complexes that facilitate transport across both membranes as has been proposed for transport of mitochondrial proteins and of ATP directly to hexokinase and creatine kinase bound to the outer membrane [31,34]. Some evidence suggests that cristae membranes are enriched in cytochrome c oxidase based upon a histochemical staining procedure involving oxidation of diaminobenzidine by cytochrome c oxidase (Fig. 4), although published studies with this procedure report conflicting results [35,36]. A definitive answer to this question will require better methods of staining specific proteins for visualization by electron microscopy/tomography. A very

Fig. 4. Mitochondria observed in situ in cerebellar cells that have been stained with diaminobenzidine (DAB) followed by conventional fixation with osmium. Cytochrome oxidase activity oxidizes DAB, producing a polymer that binds osmium; note that the crista are heavily stained, indicating they are rich in cytochrome oxidase, while the IBM is not.
promising technique is photoconversion of the ReAsH$_2$ reagent recently described by Gaietta et al. [37]. ReAsH-EDT$_2$ binds to a tetra-cysteine motif that can be genetically inserted into specific proteins. ReAsH bound to the tetra-cysteine motif is fluorescent, and excitation of fluorescence in the presence of diaminobenzidine (DAB) produces a DAB polymer that is osmophilic, yielding a highly localized deposition of osmium that is fixable by electron microscopy, presenting the possibility of localizing specific mitochondrial proteins in three-dimensional electron tomograms.

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