Review Article

Structural and Functional Roles of Non-bilayer Lipid Phase in Mitochondria

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In this review article we critically discuss the experimental data that support existence of non-bilayer lipid phase in intact mitochondria. We also review contemporary concepts on the role of non-bilayer phospholipids in controlling mitochondrial functions and remodeling. In addition, we present the latest models that propose the tentative mechanisms on how non-bilayer lipids and cardiolipin may facilitates ATP synthesis in mitochondrial cristae.

Non-bilayer lipid phase in intact mitochondria

The first report on non-bilayer lipid phase in mitochondrial membrane was made more than 40 years ago. It was revealed by ^{31}P-NMR spectroscopy that phospholipids in intact rat liver mitochondria at 37 °C coexist as bilayer and non-bilayer phases [1]. About a decade later a fraction of bovine liver mitochondrial proteolipids, which predominantly contained CL molecules bound to proteins of F_o subunits of ATP synthase, was isolated [2]. ^{31}P-NMR spectrum of this sample of mitochondrial proteolipids had a symmetrical slightly broad line shape with the signal peak at 6 ppm (hereafter called the 6 ppm signal). The conclusion was made that the 6 ppm signal derives from CL molecules which are non-bilayer arranged and immobilized by a force of attraction to the F_o subunit [2]. This conclusion was later validated by computer simulation work [3][4]. It should be noted that about a decade after the work in [2] the non-bilayer lipids arranged in hexagonal packing were discovered by small-angle neutron scattering in osmotically shocked rat heart mitochondria [5]. At about same time it was also discovered that two cytotoxins CTI and CTII from cobra venom phenocopy proteins of mitochondrial ATP synthase F_o subunit in forming protein-lipid oligomers through binding to CL molecules [6][7][8]. Cytotoxins CTI and CTII were employed to probe the structure and dynamics of cauliflower and bovine mitochondrial membranes [6][7][8]. Both samples of intact cauliflower and

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bovine mitochondria treated with CTI and CTII at 18 °C (cauliflower) or 15 °C (bovine) generated the ^{31}P-NMR spectrum with two non-bilayer signals superimposed over the lamellar signal [6][7][8]. One non-bilayer signal had a resonance at 0 ppm and another at 6 ppm. Application of DANTE (delay alternating with nutation for tailored excitation) train of saturation pulses at the high-field peak of the lamellar signal revealed that non-bilayer signal at 0 ppm derives from the lipids with rapid isotropic movement which exchange with the lipids in lamellar phase within the ^{31}P-NMR time scale, while the signal at 6 ppm derives from the lipids that do not exchange with the lipids in lamellar phase in the ^{31}P-NMR time scale [6][4]. To elucidate further details of molecular mechanism of cytotoxins interactions with mitochondria, intact mitochondria and model membranes of phospholipid composition of IMM treated separately with cytotoxins CTI and CTII were investigated with a set of biophysical methods including ^{31}P-NMR, ^{1}H-NMR, ^{2}H-NMR, EPR of oriented lipid films, luminescent quenching spectroscopy and differential scanning calorimetry [6][7][4]. It was determined that the 6 ppm signal derives from non-bilayer organized CL molecules immobilized by binding to cytotoxins and located in the intermembrane junctions between the OMM and the IMM [9]. It was also suggested that non-bilayer arranged phospholipids responsible for the 6 ppm signal could be immobilized by binding to mitochondrial proteins which have binding sites for CL similar to those of CTI and CTII [4][6][7]. Docking simulation analysis revealed that CTI and CTII share CL binding sites with the dicyclohexylcarbodiimide-binding protein (DCCD-BPF), which is the C8 rotor protein that is a part of the F_o sector embedded in IMM in bovine mitochondrial ATP synthase. The C8 rotor subunit of the F_o sector is a key element in the process of moving protons through the F_o sector [10]. Model membranes with the phospholipid composition of IMM were treated with DCCD-BPF similarly to the treatment of the same model membranes with CTI and CTII and it was demonstrated that DCCD-BPF and cytotoxins share similar membranotropic properties [4][6][7]. When DCCD-BPF was reconstituted in model membranes with phospholipid composition of IMM, the 6 ppm ^{31}P-NMR signal was observed. It was established that the 6 ppm signal derived from immobilized phospholipids that did not exchange with the phospholipids of the lamellar phase which suggested that the 6 ppm phospholipids are CL molecules immobilized by binding to DCCD-DPF [4][6][7]. It should be noted that the mobility of CL molecules immobilized by binding to DCCD-DPF or to cytotoxins is faster than mobility of annual lipids which do not generate a ^{31}P-NMR signal as they move slower than 10^{-2} - 10^{-4} s – ^{31}P-NMR time scale. This finding allowed one to employ CTI and CTII as the proteins that imitate membranotropic

effects of DCCD-DPF [11]. The further experiments have shown that at low concentrations CTI and CTII enhance ATP synthesis in intact mitochondria treated with cytotoxins and that enhancement of ATP synthesis parallels the increase in intensity of the 6 ppm signal [4][6][7][11]. Intriguingly the C8 rotor subunit of the F_o sector has been recently proposed as a key component of the mitochondrial permeability transition pore (MPTP) [12]. In this regard, one can conclude that the ability of the DCCD-BPF to generate non-bilayer lipid structures could play a key role at the formation of MPTP that could happen through the involvement of immobilized CL molecules in generation of non-bilayer structures to drive the inner and outer mitochondrial membrane fusion.

Role of non-bilayer phospholipids in mitochondrial functions and remodeling

The non-bilayer lipids, CL and phosphatidylethanolamine (PE), play essential role in dynamics and structural assembly of mitochondrial membranes [13]. In mammalian mitochondria PE constitutes about 40% of lipids in OMM and IMM, while CL constitutes about 3% and 20% of lipids in OMM and IMM respectively [14]. Both CL and PE exert asymmetrical lateral pressures in lipid polar heads and lipid hydrophobic alkyl chains areas to generate mechanical stress necessary for creating numerous folds in IMM and invaginations for cristae tubular membranes [15]. Both non-bilayer phospholipids contribute greatly not only to maintenance of functionally active states of the respiratory chain proteins, but also to overall membrane morphology of mitochondria [13].

The effects of CL and PE towards supporting structural and functional stability of IMM, such as generating membrane potential and facilitating maximal activity of electron transport chain (ETC) proteins, are different [16]. The activity of cytochrome *c* oxidase and the membrane potential of IMM decrease in lack of PE, but in lack of CL, PE exerts destabilizing effect on the respiratory chain super-complexes [16]. The stable membrane potential, efficient activity of cytochrome *c* oxidase and structural strength of the respiratory chain super-complexes requires CL [16]. CL is the main structural element in dimerization of the ATP synthases. Also, CL acts as a glue in stabilizing ribbon-like assembly of ATP synthase dimers which is a signature feature affecting overall morphology and organization of cristae membranes [17]. Apart from binding to proteins of the ETC, CL also binds to the transporters of ADP-ATP, pyruvate, phosphate carriers and to cyt *c* which cannot function efficiently without CL while deficiency of PE does not affect functioning of these proteins [18]. It is common

understanding that one of the main roles of CL is preserving elastic bonds between ATP synthase dimers and oligomers and keeping the tight associations between proteins of Respiratosomes, while PE primarily serves to supplement CL in maintaining plasticity and structural integrity of the curved area of IMM [16]. The high angle conical shape of CL with four highly flexible alkyl chains and two phosphate groups suits very well for arrangement of the tight, strong but flexible bonds between ATP synthase complexes in dimeric and oligomeric forms of the enzyme [3][4] and proteins of the respiratory chain super-complexes in the highly dynamic lipid phase of IMMs [19].

Mitochondria is an organelle which continuously changes its morphology and cristae are the highly dynamic structures in IMM which remodel their architecture in a timescale of seconds in response to changes in the energy demands depending on metabolic rates, physiological states of cells and tissues, states of health and disease of organism [20][21][22][23]. Cristae act as 'sub-organelles' connected through slit-like structures, called crista junctions, to the inner boundary membrane (Figure 1A). Electron tomography revealed that there is a dynamic interaction between cristae junction and cristae [20][24]. Disappearance of cristae and formation of new cristae, a step in cristae membrane remodeling, occurs in several seconds according to data obtained by the advanced optical microscopy [20][23][25]. Dynamics of cristae remodeling affects membrane potential of an individual crista, Ca^{2+} homeostasis, OXPHOS and apoptosis [22][23]. Cristae may act as independent bioenergetic sub-organelles each having different rates of ETC activities and ATP synthesis in different parts of the IMM [26] (Figure 1B).

ATP synthase, OPA1, MICOS, and lipid microenvironment composed mainly of CL determine the rates of cristae junction formation and remodeling of cristae [21][23][26][27]. Abnormal rates in cristae remodeling caused by CL insufficiency results in a range of diseases [22][26][28]. Creation of new cristae is facilitated by CL's non-bilayer propensity [19][27][28]. Cristae membrane bending is regulated by the MICOS complex subunits (MIC60 and MIC10), which are surrounded by CL at cristae junctions [21]. The short and long forms of OPA1 bound to CL regulate the width of cristae junction and the two phosphate groups of CL are thought to be mostly responsible for keeping proteins in cristae junctions tight [21]. It is believed that positive membrane curvature at the cristae tip is induced by ATP-synthase dimers surrounded by the CL predominant microenvironment in the inner leaflet of the cristae membrane [21]. Insufficiency in CL changes not only the cristae remodeling but also the setting of OXPHOS complexes [29] which may cause cancer, cardiovascular pathologies and

neurodegeneration [20][30]. The abnormally swollen and highly interconnected cristae induced by the decline in CL molecules [31][32] lead to defects in heart and skeletal musculature [31].

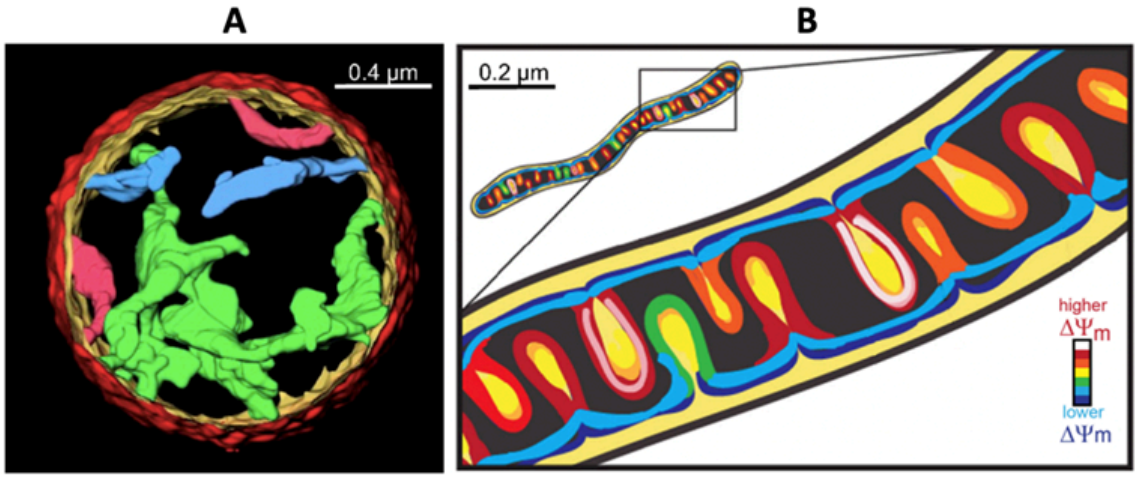

Figure 1. A – Tomographic reconstruction of a rat liver mitochondrion displaying the outer membrane (red), the inner boundary membrane (yellow) and different cristae (green, blue and magenta), which have one or more narrow tubular connections at the inner membrane (courtesy of Dr. Carmen Mannella, The Wadsworth Center, Albany, NY / University of Maryland School of Medicine, Baltimore, MD). Tomography was performed on a sample prepared by conventional chemical fixation; but very similar images were obtained by cryo-electron tomography. **B** – Model showing the heterogeneity of the mitochondrial membrane potential based on high-resolution light microscopy imaging of living cells [33]. Variations of the membrane potential in between the inner boundary membranes and different crista membranes are illustrated by false colors. This figure is modified from [34].

Intermembrane exchange, membrane fusion and fission are the central processes in the remodeling of cristae architecture [32][35][36][37]. This is evident from the multiple junctions to the inner boundary membrane and numerous cristae-membrane interconnections (Figure 1A). Decline in mitochondrial CL caused by aging and diseases inhibits dynamics of IMM fusion and fission which leads to defects in cardiac and skeletal muscles [31] and cardiovascular and neurological disorders and cancer [31][37]. Reduced levels of CL in IMM reduces membrane coupling and efficiency of ATP synthase and respiratory ETC in muscle cells [38].

It has been well established that CL, a lipid in IMM with the highest non-bilayer propensity, drives the membrane fusion and fission via triggering lipid phase transitions from bilayer to non-bilayer

phase [3][4][6][7][8][11][39][40]. ^{31}P-NMR spectroscopy observes dynamics of phospholipid phase polymorphism in model and native IMM in the timescale of 10^{-2} to 10^{-4} s [6][7][11]. The above-mentioned experimental data along with the recent review that stresses the role of non-bilayer lipids in mitochondrial architecture and functions [41] strongly suggest that the high inclination of CL to form non-bilayer phase in IMM is a key driving force that promotes rapid cristae remodeling in a timescale of seconds.

Non-bilayer lipid, cardiolipin, facilitates ATP synthesis in cristae

An individual crista is a self-sufficient bioenergetic sub-organelle of mitochondria that serves as a suitable surface area for the clustering of ETC proteins and ATP synthase dimers which retain protons on the inner surfaces of crista membranes [9]. Binding of CL presumably on the inner surface of crista membrane to the F_o sector of ATP synthase creates the ATP synthase dimers in the apex of crista with the highest membrane surface curvature in cristae. The CL mediated dimerization of ATP synthases along the long axis of crista creates the membrane raft-like structures with the high membrane surface curvature at the peak of the raft [9]. Each ATP synthase complex of the dimer sits on opposite sides of the raft's peak (Figure 2 A) [9]. Clustering of OXPHOS oligomers both in the apex of cristae and at the peaks of membrane rafts, which is mediated by CL binding to OXPHOS proteins, creates optimal conditions for kinetic coupling of the ETC proteins with ATP synthase in which the ETC proteins transfer protons along the inner cristae membrane surface to the ATP synthase dimers [9][42][43].

The idea of protons diffusing between the ETC proteins along the inner surface of crista membrane was first suggested in 1961 [44] and it was convincingly corroborated one and half decades later in the octane-water interface system [45]. About two decades later the existence of a kinetic barrier for proton transfer from a membrane surface to bulk water was reposted [46] and a few more years later a proton transfer across the interface under conditions of catalysis and driven by the proton gradient on the membrane surface was demonstrated [47]. Four more years later a metastable bond between protons and mitoplast surface [48] and protons from Brønsted acids bounded to mitochondrial surface and serving as a substrate for ATP synthase were reported [49]. In a period from five to two years ago a few more important studies were published. It was shown that ATP synthesis rate is determined by lateral movement of protons along the membrane surface from proton pumps to ATP synthase [50] and that ATP synthesis in cristae membranes is driven by kinetic coupling of the ETC

with ATP synthase, but not by proton gradient in bulk water [42], and finally it was suggested that ATP synthesis is driven by proton currents inside the coupling membrane [51]. The last study is of a particular interest. We suggest that protons inside the coupling membrane could be found on the inner surface of inverted micelle formed in a membrane bilayer same as suggested for inverted micelle with cytotoxin in its inner surface. Due to the high structural tension, inverted micelle releases protons along the concentration gradient of protons to the membrane surface on matrix side from where protons are returned back to the inter-crista space via proton pumps. When inverted micelle releases protons inverted micelle transforms back to bilayer membrane. Then when protons diffuse again along membrane surface back to ATP synthase surrounded by CL, conical shape of CL increases to trigger formation of inverted micelle with protons in the inner surface of micelle which then again releases protons to the matrix. It is quite possible that the reversible polymorphic transitions from bilayer membrane to non-bilayer micelle near the ATP synthase trigger rotation of the ATP rotor needed for ATP synthesis. Should the suggested mechanism of proton currents inside an inverted micelle that move protons across the coupling membrane down the proton concentration gradient be proven correct, this would be another evidence demonstrating a central role of non-bilayer lipid phase in ATP synthesis in cristae membranes. We believe that kinetic coupling of the ETC with ATP synthase makes a lot of sense. That is because protons move along inner surface of crista membrane over ETC proteins to ATP synthase and get transferred across crista membrane inside inverted CL micelle and then returned back via proton pumps to the inner surface of crista membrane. Thus, in kinetic coupling the movement of protons along the crista membrane surface and across the crista membrane does not cause a fluctuation in pH in bulk waters across the crista membrane. We see two weak points in the chemiosmotic theory driven by proton gradient in bulk solutions across the crista membrane. First, it would take a colossal number of H^+ ions in solutions in intermembrane space and matrix to create a proton gradient across cristae membranes. Second, ATP synthesis coupled with movement of protons from bulk solutions across crista membrane would create pH fluctuations in solutions in intermembrane space and matrix which would affect 3D folding and functions of proteins and other organic substances in solutions.

The reason for ATP synthases to get arranged in dimers is to increase curvature on the inner side surface of crista membrane. ATP synthase dimerization creates maximal membrane curvature in the apex of crista and at the peaks of rafts that leads to the electric charge redistribution in which protons are pushed into the region of maximum curvature where ATP synthase dimers are located [34][9]. This

boosts the density of protons near the F_O subunit and increases the rate of proton translocation to matrix and the rate of ATP synthesis [34]. The boost in proton density enhances neutralization of phosphate groups of CL that increases the conical shape of CL and membrane curvature and eventually turns CL in the lamellar phase to the non-bilayer CL inverted micelle which transports protons along concentration gradient to the matrix side of membrane. As we suggested above, formation of the CL inverted micelles may trigger rotation of ATP synthase rotor. It has been recently reported that CL selectively binds to the conserved lysine residues in the ATP synthase rotor [52]. It is possible that the high proton density weakens the CL bond with lysine residue which triggers both the rotation of ATP synthase rotor and formation of the CL inverted micelles. Decrease in proton density turns the CL inverted micelles to the lamellar phase to reestablish the CL bond with lysine residues which in turn stops the rotation of the ATP synthase rotor. This could be seen as a dynamic equilibrium linking the rate of lipid polymorphism in cristae membrane and thus OXPHOS efficiency on one side of equilibrium and the cristae membrane tranquility in lamellar phase on another side. A position of equilibrium is controlled by the energy demand that changes with the change in the physiological state.

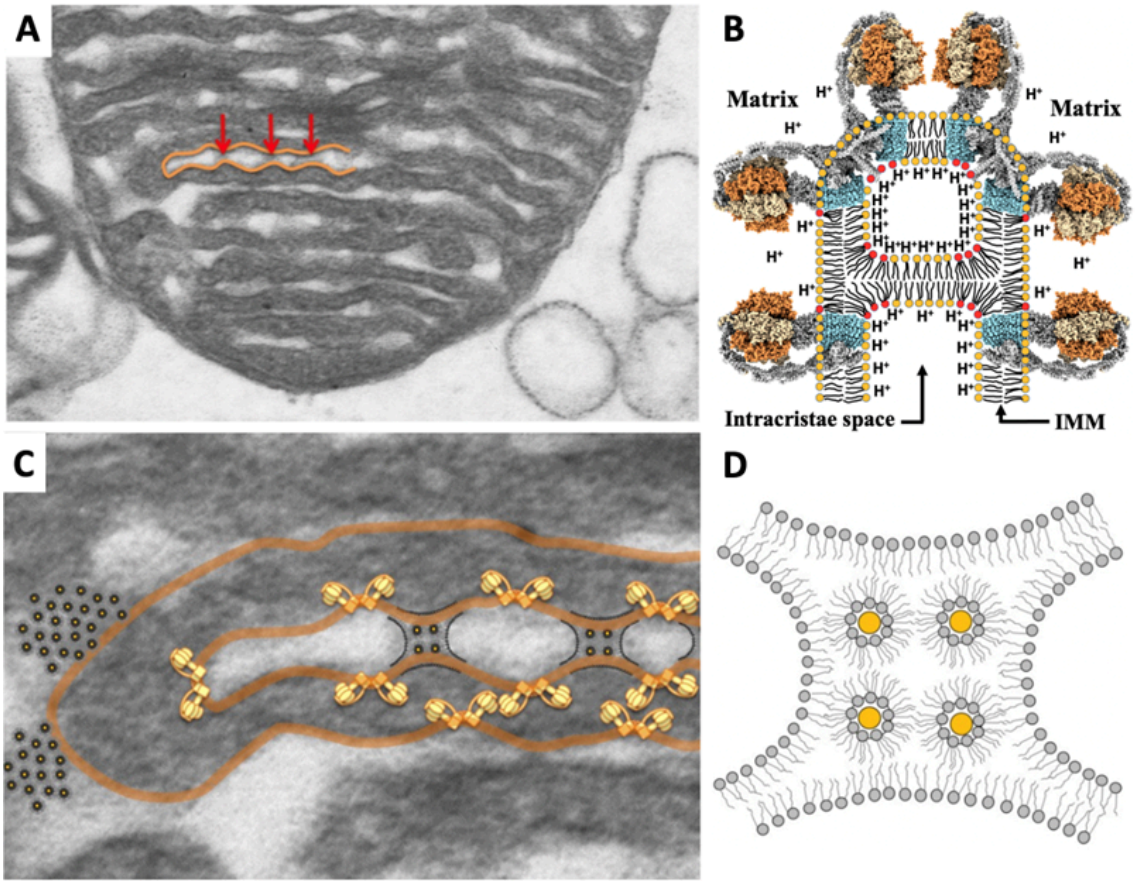

Figure 2. A – Electron microphotography of rat heart mitochondria ultra-thin slice [53]. Tentative intermembrane junctions with non-bilayer inverted micelles in the intra-crista space as described in [7] [11][54] indicated by red arrows. **B** – The apex of crista with high surface curvature is formed at CL mediated dimerization of ATP synthases. Formation of intermembrane junction mediated by CL polymorphism [7] [54] is a key step in creation of compartment which increases H^+ concentration along inner surface. The alpha and betta subunits of ATP synthase are shown in yellow and orange respectively, the c ring in blue, other subunits in grey, polar head of CL in red and that of other lipids in yellow. Alkyl chains are not drawn over F_0 subunits. **C** – Magnified fragment of crista from above image with proposed positions of the ATP synthase dimers and the intermembrane junctions. **D** – A model of intermembrane junction induced by formation of CL-made inverted micelles with a cationic protein in center of micelles shown in orange. All models and drawings are schematic, and scales and positions are not strictly sustained. This figure is modified from [34][54].

It has been demonstrated in recent studies that creating compartments in the intra-crista space by forming intermembrane junctions between inner surfaces of parallel crista membranes (Figure 12, B)

may further facilitate ATP synthesis in crista as protons are ‘squeezed’ along the inner membrane surface of compartments to further increase H^+ ions density near the F_o subunits to further promote transport of protons into matrix and to further increase ATP synthesis [34][7][54].

The initial step in formation of intermembrane junction in the intra-crista space, which is likely created between two areas of maximum curvature protruding into the intra-crista space, is driven by attraction of CL molecules on the internal sides of parallel membranes of cristae to a cationic peptide on the surface of opposite membrane of crista via a mechanism described previously [7][11][34][54]. A cationic protein which serves as the initial point of intermembrane contact becomes the center of inverted micells which due to the high tension from membrane curvature may be transformed to the bilayer intermembrane junction and release cationic protein into intra-crista space. CLs are found on the surface of lipid layers with high curvature (Figure 12, B). When a thick intermembrane junction is created, many inverted micelles with a cationic protein in their center could be formed (Figuree 12, C, D) to make a stabler intermembrane junction than the one is made of a single bilayer (Figure 12, B). Cationic proteins that may initiate formation of intermembrane junction could be creatine kinase, cyt *c*, or even misfolded DCCD-BPF not incorporated into ATP synthase c-rings [34]. Intermembrane junctions may also serve to maintain appropriate space inside of crista by preventing adjacent cristae membranes from being squeezed toward each other. Appropriate intra-crista space is needed for transport of nucleotides, phosphate group and nucleotide carriers and substrates in the bulk solution. A high dynamics of intermembrane junctions which undergo bilayer to non-bilayer transitions should be appreciated as they change reversibly in response to changes in proton density on the inner crista membrane surface. At high proton density bilayer phase transforms to non-bilayer and at low proton density a reverse process takes place [34]. Due to reversibility of the process, intermembrane junctions do not interfere with the transport of substrates and nucleotides in the bulk solution in intra-crista space. It has been also proposed that a channel linking mitochondrial matrix on opposite sides of the crista may be created by fusion of cristae membranes triggered by non-bilayer junctions [34]. Similar events may take place when the lamellar structure of cristae transforms to the tubular structure.

Conclusions

The analysis in this review of extensive experimental and theoretical work on non-bilayer lipid phases in model and mitochondrial membranes that is available up today allows one to conclude that the non-bilayer phases are essential parts of fully functional mitochondrial membranes and that the non-

bilayer phases are not only indispensable elements of maintaining the structural dynamics and remodeling of inner mitochondrial membranes, but may also be a centerpiece in mechanism of ATP production. Concepts related to the roles of the non-bilayer phases that have been reported in IMMs and presented here are still in the process of development and require further research for confirmations. We believe this review will attract more attention to the roles of non-bilayer phases in energy transducing membranes and will facilitate more rigorous research on elucidation of new important details in mechanisms of fundamental bioenergetic systems such as thylakoid and inner mitochondrial membranes.

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