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Non-bilayer structures in mitochondrial membranes regulate ATP synthase activity

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ABSTRACT

Cardiolipin (CL) is an anionic phospholipid at the inner mitochondrial membrane (IMM) that facilitates the formation of transient non-bilayer (non-lamellar) structures to maintain mitochondrial integrity. CL modulates mitochondrial functions including ATP synthesis. However, the biophysical mechanisms by which CL generates non-lamellar structures and the extent to which these structures contribute to ATP synthesis remain unknown. We hypothesized that CL and ATP synthase facilitate the formation of non-bilayer structures at the IMM to stimulate ATP synthesis. By using ^{1}H NMR and ^{31}P NMR techniques, we observed that increasing the temperature (8 °C to 37 °C), lowering the pH (3.0), or incubating intact mitochondria with CTII - an IMM-targeted toxin that increases the formation of immobilized non-bilayer structures - elevated the formation of non-bilayer structures to stimulate ATP synthesis. The F_0 sector of the ATP synthase complex can facilitate the formation of non-bilayer structures as incubating model membranes enriched with IMM-specific phospholipids with exogenous DCCD-binding protein of the F_0 sector (DCCD-BPF) elevated the formation of immobilized non-bilayer structures to a similar manner as CTII. Native PAGE assays revealed that CL, but not other anionic phospholipids, specifically binds to DCCD-BPF to promote the formation of stable lipid-protein complexes. Mechanistically, molecular docking studies identified two lipid binding sites for CL in DCCD-BPF. We propose a new model of ATP synthase regulation in which CL mediates the formation of non-bilayer structures that serve to cluster protons and ATP synthase complexes as a mechanism to enhance proton translocation to the F_0 sector, and thereby increase ATP synthesis.

1. Introduction

The ATP synthase complex is a large multimeric enzyme complex comprised of over 20 subunits. More than half of the subunits that form the F_0 sector of the ATP synthase complex are submerged within the inner membrane of mitochondria (IMM) [1,2]. The transport of H^+ through the F_0 sector of the ATP synthase complex is generated by establishing and maintaining a transmembrane proton gradient [1,2]. ATP synthesis mediated by the ATP synthase complex is accomplished via the rotation of the central stalk and a ring of c-subunits (outer c subunits in bovine ATP synthase) in the membrane domain [3]. The active form of ATP synthase complex consist of a dimer that is maintained via a "dimerization interface" whereas the monomer form of ATP synthase is inactive [4,5]. These dimers of ATP synthase have been localized at the apex of cristae, a site that serves as a proton trap to increase efficiency of ATP synthase [4]. Moreover, ATP synthase can undergo multiple conformations by forming large oligomers that consist of rows of dimers imbedded within cristae via an "oligomerization interface" to remodel cristae morphology. Interestingly, neither cristae morphology nor the energetic status of mitochondria govern dimerization or oligomerization of ATP synthase suggesting the existence of unidentified biophysical mechanisms [6,7] that maintain the active conformation of ATP synthase. Based on the recent high resolution X-ray crystal structural data, the F_0 sector of ATP synthase is comprised of c subunits (8 c subunits in bovine or 10 in yeast ATP synthase) radially arranged around the stalk structure [3,8–10]. Importantly, the crystal structure of the F_0 c-ring has been solved by different research groups. For instance, in yeast, the high resolution crystal structure of the c-ring consists of 10C subunits assembled in an hour-glass shaped structure that harbors a strong hydrophobic center. One of the subunits (subunit

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9 in yeast) identified as dicyclohexylcarbodiimide (DCCD)-binding protein (DCCD-BPF) is involved in proton translocation through the F_0 c-ring of the ATP synthase [11]. The inner and outer rings of the N-terminal and C-terminal α-helices are maintained by hydrophobic interactions and make few interactions with water [12,13]. Moreover, each C subunit of the F_0 consist of four α-helical fragments that contains a strong hydrophobic core. The C subunit of F_0 is a hairpin containing two transmembrane-helices, connected by a short, partially ordered loop. The first two helices form the N-terminal transmembrane domain whereas the last two helixes from the C-terminal helix traverses the phospholipid membrane to anchor the protein to the IMM [12].

There is evidence that suggest the existence of non-bilayer structures - small lipid structures that exist in a non-lamellar phase - in specific regions of the IMM. These non-bilayer structures are required to maintain mitochondrial structural integrity and essential mitochondrial functions including regulating the calcium uniporter, transacylation reactions of phospholipids, and proton translocation to the F_0 sector of ATP synthase [14–17]. Importantly, non-bilayer structures have been shown to exist in organelles under physiological conditions to drive critical enzymatic reactions within specialized microdomains [16,18].

Cardiolipin (CL), an anionic non-bilayer phospholipid, is required to maintain normal mitochondrial structure, promote mitochondrial fusion, and stimulate oxidative phosphorylation [19,20]. In addition, CL can also act as a proton trap to shuttle protons to the ATP synthase complex to regulate ATP synthesis [2,21]. Furthermore, CL, but not other anionic phospholipid, can mediate the formation of non-bilayer structures in mitochondria, presumably via its interaction with mitochondrial proteins [22]. Phosphatidylethanolamine (PE), a major neutral mitochondrial phospholipid, tends to form hexagonal phase structures, a particular form of non-bilayer structures. However, to date, it is not known if a structural polymorphism of PE is mediated via its interaction with mitochondrial proteins.

Although the structural organization of the ATP synthase complex and the molecular mechanisms by which the ATP synthase complex produces ATP have been well characterized [1], it is not known how non-bilayer structures, CL and the ATP synthase interplay to regulate mitochondrial functions (e.g. ATP synthesis).

Here, we hypothesized that CL interacts with the F_0 sector of ATP synthase to facilitate the formation of transient non-bilayer structures in the IMM as a mechanism to enhance ATP levels in mitochondria. Overall, by using a myriad of biophysical techniques, our data show that immobile non-bilayer structures in mitochondria govern ATP synthesis via the following biophysical mechanism. First, an elevation of the proton gradient leads to a lower pH at the IMM space which destabilizes the bilayer packing of acidic phospholipids and induces lipid phase transitions (from a lamellar to a non-bilayer structure) within specific regions of the IMM that are in close proximity to the DCCD-BPF (C subunit) of the F_0 sector. Mechanistically, CL, but not other anionic phospholipids, binds to the F_0 sector of ATP synthase to facilitate the transition from a bilayer to non-bilayer phase of immobilized phospholipids in the IMM. These immobile non-bilayer structures, presumably an inverted micelle or similar structures, are organized in such a way that favors both the oligomerization of the F_0 sector and act as a specialized proton trap to shuttle protons to the ATP synthase complex leading to enhanced ATP synthesis.

2. Materials and methods

2.1. Reagents

Egg yolk L-α-phosphatidylcholine (PC), cardiolipin (CL) from *E.coli*, bovine brain L-α-phosphatidyl-L-serine (PS), egg yolk L-α-phosphatidic acid (PA), and potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO). All phospholipids were further purified on silica columns. Central Asian cobra *Naja naja oxiana* crude venom was obtained as a gift from Prof. L.Ya. Yukelson (Institute of Biochemistry, Uzbekistan Academy of Sciences) in lyophilized form. Cardiotoxins CTII and CTI, also known as cytotoxin CTII (or Vc5) and cytotoxin CTI (or Vc1) respectively, were isolated from 500 mg of crude venom as previously published [23] and were further purified by cation exchange HPLC by using a SCX 83-C-13-ET1 Hydropore column as previously described [24]. The purity of CTII and its molecular weight were assessed by Native-PAGE (Fig. 5C). The molecular weight of CTII is approximately 7 kDa. Based on the Native-PAGE assay, the purity of "gradient-purified" CTII is high (~99%) based on the absence of noticeable contaminating protein bands (Fig. 5C, lane B). All other reagents were from Sigma-Aldridge (Moscow, Russia).

2.2. Preparation of isolated mitochondria and exogenous DCCD-binding protein

Bovine heart mitochondria were isolated as previously described using sequential centrifugation steps [14,25]. In brief, the final crude mitochondrial pellet was re-suspended in 5 ml of washing medium (0.3 M mannitol, 10 mM MOPS, 1 mM EDTA, and 0.1% (w/v) BSA at pH 7.4). The re-suspended mitochondria were osmotically shocked with 5 ml of 20% (w/v) sodium succinate in washing medium for 10 min. to release water soluble, phosphate-containing molecules of non-phospholipid nature. This step is necessary to simplify the ^{31}P NMR spectra, thereby facilitating the analyses of the organization and molecular mobility of mitochondrial bilayers. The osmotically-shocked mitochondria were allowed to recover in an isotonic environment by immediately diluting the mitochondria in 40 ml of ice-cold washing medium (0.3 M mannitol, 10 mM MOPS, 1 mM EDTA, and 0.1% (w/v) BSA at pH 7.4), followed by centrifugation at 11,000 × *g* for 10 min. The supernatant was then decanted and the mitochondrial pellet was re-suspended in 5 ml of washing medium to a final concentration of 60 mg/ml which was sufficient for three independent ^{31}P NMR experiments. Following an incubation of 20 min, 1.5 mM sodium succinate was added to 0.1 ml of re-suspended mitochondria as a substrate for oxidative phosphorylation, and quality control tests were performed in order to assess for the functional quality of mitochondria. In brief, respiratory control index (RCI) ratios of mitochondria recovered in isotonic medium (0.3 M mannitol, 10 mM MOPS, 1 mM EDTA, and 0.1% (w/v) BSA at pH 7.4) after resuspension, were assayed in the presence of 1.5 mM succinate as a substrate for complex II. By using a Clark electrode, RCI was then measured as the ratio of the O_2 consumption rate following the addition of 4 mM ADP and Pi divided by the O_2 consumption rate after all ADP has been converted to ATP. The RCI values for untreated mitochondria (baseline) were observed to be between 3 and 4, a range that is consistent with high quality, functional mitochondria [26]. Moreover, this result suggest that mitochondria are highly coupled (oxidative phosphorylation and ATP synthesis), even after subjecting mitochondria to osmotic shock as previously reported [22].

Mitochondrial samples (derived from the same stock: 1.5 ml of mitochondria in washing buffer with a protein concentration of 60 mg/ml and phospholipid concentration 6.3×10^{-2} M) were subjected to different treatments. For data shown in Fig. 1, mitochondria were incubated at increasing temperatures within their NMR tubes while their ^{31}P NMR signals were allowed to accumulate for 30 min (Fig. 1). To manipulate the amount of non-bilayer structures that are generated in mitochondria, mitochondria were treated with cardiotoxin II (9×10^{-4} M CTII), cardiotoxin I (9×10^{-4} M CTI), or with cobra venom phospholipase A_2 (PLA_2, 1×10^{-7} M PLA_2) at 15 °C in a control sample (Fig. 2). Immediately after NMR spectra were recorded, ATP content (amount of ATP residing in mitochondria, not the rate of ATP synthesis) in mitochondrial samples treated with CTII or CTI and/or PLA_2 and in untreated sample controls was measured using a protocol as previously described to assess mitochondrial content [27] with minor modifications. To stop further ATP synthesis, 0.5 ml of mitochondria taken from NMR tube were lysed by 20 min incubation in