**SHORT REPORT**

**Atomistic Simulations Indicate the c-Subunit Ring of the F1Fo ATP Synthase   
is Not the Mitochondrial Permeability Transition Pore**

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**ABSTRACT**

Pathological metabolic conditions such as ischemia induce the rupture of the mitochondrial envelope and the release of pro-apoptotic proteins, leading to cell death. At the onset of this process, the inner mitochondrial membrane becomes depolarized and permeable to osmolytes, due to the opening of a non-selective protein channel of unknown molecular identity. A recent study purports that this channel, referred to as Mitochondrial Permeability Transition Pore (MPTP), is the lumen of the c-subunit ring of the ATP synthase, upon dissociation from the catalytic domain. Here, we examine this claim for two c-rings of different lumen width, through calculations of their ion conductance and selectivity based on all-atom molecular dynamics simulations. We also evaluate the likelihood that this lumen is hydrated rather than empty or blocked by lipid molecules. These calculations demonstrate that the properties of the lumen of a correctly assembled c-ring are inconsistent with those attributed to the MPTP.

**INTRODUCTION**

Under certain pathological conditions mitochondria cease ATP production and instead trigger apoptosis. At the onset of this process is the depolarization of the inner mitochondrial membrane, which under normal conditions sustains the proton-motive-force powering the ATP synthase. It has long been hypothesized that this depolarization is mediated by a channel protein referred to as the Mitochondrial Permeability Transition Pore (MPTP)1-4. The hallmark properties of this putative channel are: very large ionic conductance (up to 1300 pS); high permeability to small molecules, including osmolytes (less than 1.5 kDa); and no solute specificity (aside from size-limit) or ion selectivity. Thus, opening of the MPTP, thought to be induced by e.g. excessive amounts of Ca2+ or ROS in the mitochondrial matrix, is catastrophic for the cell: not only does it short-circuit the respiratory chain, causing the ATP synthase to reverse its activity and deplete cellular ATP, but it also induces the osmotic swelling of the matrix, rupturing the outer membrane and causing the release of pro-apoptotic proteins3.

The molecular identity of the MPTP remains to be established. Seemingly promising studies have implicated mitochondrial proteins as diverse as the outer-membrane Voltage Dependent Anion Channel (VDAC), the inner-membrane Adenine Nucleotide Translocase (ANT), and the non-transmembrane cyclophilin D; ultimately, however, none of these proteins was found to be actually essential2-5. More recently, Jonas and co-workers have proposed that a sub-complex of the mitochondrial F1Fo ATP synthase, known as the c-ring, is the MPTP6. The c-ring is an oligomer of identical copies of subunit-c, each of which is a hairpin of two transmembrane helices (**Fig. 1 – Supp. 1A**); when assembled, these helices are arranged in two concentric rings around a central lumen7-9. The F1 domain, which projects out of the membrane and contains the catalytic domain, sits atop the ring (**Fig. 1 – Supp. 1B**)10-11. In the operating enzyme, the membrane Fo domain works like a turbine, with the c-ring rotating against other static elements as protons sequentially bind to each of the c-subunits and are released across the membrane after a revolution12-13. These proton-binding sites are on the outer surface of the ring, and feature a conserved glutamate and other polar groups (**Fig. 1 – Supp. 1A**)8-9, 14-15; the lumen, by contrast, is largely hydrophobic. What Jonas et al. propose is that the metabolic conditions associated with the mitochondrial permeability transition induce the F1 and Fo sectors to dissociate, upon which the lumen of the c-ring would become the MPTP6.

Here, we use all-atom molecular dynamics simulations to directly investigate the plausibility of this claim, which has been questioned by others5, 16. Specifically, we consider two c-rings of different size, and examine their ion-conducting properties under the assumption that their lumen is hydrated. Independently, we examine the validity of this assumption, i.e. we evaluate whether a hydrated lumen, which is a requisite for ion/solute permeation, is more or less probable than an empty lumen or a lumen occupied by a lipid bilayer. The results strongly suggest that the c-subunit ring, if correctly folded and assembled, is not the elusive MPTP, even if the F1 sector did in fact became detached from the membrane domain.

**RESULTS AND DISCUSSION**

**The hydrated c-ring lumen does not conduct like the MPTP**

We first considered the c10-ring from *S. cerevisiae*, which is the only mitochondrial c-ring of known atomic structure (**Fig. 1A**)9, 15. The lumen of this ring is also wider than that of the c8-rings found in vertebrates (**Fig. 1 – Supp. 2A**)11, and thus it is hypothetically more likely to sustain a larger conductance. (Note the permeability transition also occurs in yeast mitochondria4.) To evaluate the conducting properties of this c10-ring, we calculated the free-energy profile associated with the permeation of either K+ or Cl- across the lumen, as well as the diffusion-coefficient profiles for each ion (**Fig. 1B-D**), in a state in which the c-ring lumen is filled with water at bulk-like density (as will be discussed below, this state is intrinsically metastable though not the most probable). The resulting conductance values are 2.5 ps for K+ and 116 ps for Cl- (for 100 mM KCl). These values are clearly inconsistent with the properties of the MPTP, not only in terms of their magnitude, but also in that they reveal a marked anion selectivity. Analogous calculations carried out in the absence of electrolytes indicate that this selectivity owes to a ring of arginine side-chains at the mouth of the lumen, on the matrix side, which influences the energetics and dynamics of ion permeation electrostatically (**Fig. 1G, 1A**). This electropositive arginine ring, which is conserved in other mitochondrial c-rings (**Fig. 1 – Supp. 2A**), counters the cost of dehydration for Cl-, but imposes an additional barrier for K+ (**Fig. 1C**), incompatible with the high conductance properties of the MPTP.

**The lumen of the mitochondrial c-ring is not hydrated**

Because the interior surface of the c-ring is highly hydrophobic, the permeation of ions and osmolytes across the lumen requires an aqueous pathway. Thus, another way to evaluate the notion that the c-ring is the MPTP is to determine whether this lumen is indeed likely to be filled with water. We therefore sought to calculate the free-energy gain or cost associated with varying the density of water inside this region. To do so, we developed a variation of the Metadynamics enhanced-sampling technique, with which we simulated multiple transitions between an empty state of the c-ring lumen and another that is maximally hydrated, while quantifying the energetics of this process (**Methods**). The resulting free-energy profile (**Fig. 2A**) reveals that a metastable state does exist in which the lumen of the mitochondrial c-ring is filled with water, at bulk-like density (**Fig. 2B**). However, this water-filled state is approximately 15 kcal/mol less favorable than a second state in which most of the lumen of the c-ring is void of any water (**Fig. 2B**). That is, the probability that the c-ring lumen exists in a ‘conducting state’ is negligible – a dehydrated, non-conducting state is much more probable, by multiple orders of magnitude.

**The lumen of the mitochondrial c-ring is plugged by lipid molecules**

Irrespective of the question of hydration, the notion that the c-ring is the MPTP implicitly assumes that lipid molecules are somehow excluded from its lumen as c-subunits gradually assemble to form a ring. There is, however, no evidence that this is the case; to the contrary, detergent molecules are often observed in the lumen in X-ray structures of c-rings17-19, and AFM images of two-dimensional ring arrays also indicate the lumen might be occluded20. To conclusively evaluate this question, we carried out 3 independent simulations in which the mitochondrial c-ring lacked two of the c-subunits (**Fig. 3**). This open c-ring resembles the kind of assembly intermediate that has been observed in AFM micrographs of prokaryotic c-rings21-22. Initially, we assumed the lumen of the c-ring to be hydrated (**Fig. 2B**), as this is the necessary feature of the hypothesized conducting form. As shown in **Fig. 3A-C**, however, the 3 simulations consistently demonstrate that all this water is quickly displaced by lipid molecules gradually entering the c-ring lumen; ultimately, these lipid molecules occupy the totality of the pore, in a bilayer-like arrangement akin to that outside the c-ring (**Fig. 3C**). There is no reason to assume that the incorporation of the two missing c-subunits would alter the lipid occupancy of the lumen, which would therefore remain in a non-conducting state when the c-ring assembles completely.

**Enlargement of c-ring lumen does not explain properties of MPTP**

One of the elements in the proposal that the mitochondrial c-ring is the MPTP is that the size of lumen might expand upon dissociation of the F1 sector6. This expansion is not entirely implausible; indeed, subtle variations in the amino-acid sequence of the c-subunits can destabilize the native state of a c-ring, and favor the assembly of larger c-rings with a slightly greater number of c-subunits23. With this in mind, we repeated all calculations presented thus far for a variant of the c13-ring from the bacterium *B. pseudofirmus* (**Fig. 1E-F**). Among the ATP synthase c-rings of known structure, this specific c13-ring features the widest lumen24. Thus, although no process akin to the permeability transition has been described in bacteria, we reasoned this ring would be a suitable system to assess whether an expanded mitochondrial c-ring might be the MPTP. For a better comparison, two of the residues lining the lumen of the ring were mutated, to make it more similar to the mitochondrial c-ring (**Fig. 1 – Supp. 2BC**). The results from this new set of calculations, however, confirm the conclusions drawn above. Because the lumen is wider, the permeability of the hydrated state is significantly larger, but this state remains highly anion-selective (**Fig. 1H**), unlike the MPTP. Moreover, this water-filled state is still disfavored, relative to the empty lumen, albeit to a lesser degree than in the c10-ring (**Fig. 2CD**). Similarly, though, independent simulations of an assembly intermediate lacking two c-subunits demonstrate that the preferred state is one in which lipid molecules occlude the lumen, in a bilayer arrangement (**Fig. 3D-F**).

**CONCLUSIONS**

This study demonstrates that the characteristics of the lumen of the mitochondrial c-ring are incompatible with the conducting properties of the MPTP. Consistent with the fact that the F1 domain does not actually seal the entrance to the lumen of the c-ring when in the operating enzyme (**Fig. 1 – Supp. 1C**), the interior of the ring is unlikely to be water-filled, i.e. it is in a non-conducting state, thereby precluding the dissipation of the ion-motive-force that powers ATP synthesis. Our results also show that even if the c-ring became dissociated from the F1 domain6, despite the nanomolar affinity of this interaction25, and even if its lumen was to become hydrated, under some hypothetical *in vitro* conditions, the conductance levels sustained by the c-ring would be unlike those of the MPTP.

Admittedly, this study does not rule out that an as-yet-unknown molecular structure consisting of c-subunits, distinct from that of the c-ring, matches the properties of the MPTP. However, it is also worth noting that no evidence of such alternative structure exists, to our knowledge. Structural studies of the c-subunit and its oligomers date back two decades; these studies include two- and three-dimensional crystallography, liquid and solid-state NMR spectroscopy, atomic-force microscopy, and more recently, single-particle cryo-electron microscopy12-13,26. Across a wide variety of species and experimental conditions, the consistent conclusion from these studies is that the c-subunits assemble in ring-like oligomers such as those analyzed here. This conclusion holds true for wild-type, mutagenized and inhibited forms of the c-ring, and for a variety of expression systems (including cell-free). Moreover, to our knowledge no apparent discrepancy exists between this set of structural data and the even larger collection of biochemical and functional measurements gathered for this enzyme or its constituent elements. Indeed, in the instances where clear thermodynamic data has been attained, e.g. on Na+/H+ binding to isolated c-rings, it was found to be not only quantitatively consistent with kinetic measurements for the complete enzyme functioning in membranes, but also explicable based on existing structures and theoretical considerations27-28. In absence of concrete experimental evidence of the existence of an alternative architecture, the logical conclusion from this body of work and the present study is therefore that the c-ring from the mitochondrial ATP synthase is not the MPTP.

**ACKNOWLEDGEMENTS**

This work was funded by the Division of Intramural Research of the National Heart, Lung and Blood Institute, National Institutes of Health.

**MATERIALS AND METHODS**

**Simulation systems and general specifications *–*** The molecular dynamics (MD) simulation systems used to study the mitochondrial c10-ring from *Saccharomyces cerevisiae* were adapted from a system used in earlier studies of the proton-binding sites in this structure9, 15. Briefly, the c10-ring had been embedded in a hydrated (and pre-equilibrated) palmitoyl-oleoyl-phosphatydyl-choline (POPC) lipid bilayer, using GRIFFIN29, and equilibrated extensively through a series of restrained and unrestrained MD simulations. Here, the 4 lipid molecules occupying the center of the c-ring were removed, and instead the lumen was initially filled with water molecules. Several alternative systems were prepared: for the complete ring, one system included 100 mM KCl, while the other included counter-ions only; a simulation system was also prepared in which the c-ring lacks two c-subunits, thus resembling an assembly intermediate. After making these changes, energy minimizations, restrained and unrestrained molecular dynamics simulations were used to equilibrate these systems.

The simulation systems for the c13-ring from *Bacillus pseudofirmus* OF4 were also adapted from a system used in a previous study of its proton-binding sites30. The central lumen of this ring is about 2-fold wider than that of the mitochondrial c10 ring, but the amino-acid composition of the protein surface lining this lumen differs. Thus, to make the c13-ring more similar, we introduced two mutations (K26L and E30Q) at the entrance of the lumen, on the side that would be exposed to the mitochondrial interior or matrix. In addition, the POPC lipid molecules inside the central lumen were also replaced by water. The resulting systems (with and without added 100 mM KCl, for the complete c13-ring, plus a system missing two c-subunits) were equilibrated analogously to the c10-ring systems.

All MD simulations were performed with NAMD231, using the CHARMM36 forcefield32-34, at constant temperature (298 K) and pressure (1 atm) and with periodic boundary conditions in all directions. Long-range electrostatic interactions were calculated using the Particle-Mesh-Ewald algorithm, with a real-space cut-off of 12 Å. Van der Waals interactions were modeled with a Lennard-Jones potential, cut-off at 12 Å using a smooth switching function taking effect at a distance of 10 Å.

**Ion conductance calculations *–*** Following Hummer and co-workers35, the conductance of the c10-ring lumen was calculated using the expression:

where *q* is the charge of the permeant ion, *C* is the bulk ion concentration (100 mM), *S* is the effective cross-section area of the lumen (201 Å2 for c10-ring and 616 Å2 for c13-ring), *k*B is the Boltzmann constant and *T* the temperature. is the one-dimensional free-energy landscape reflecting the translocation of the ion across the protein, and is the position-dependent diffusion coefficient of the ion along the lumen; both and were calculated using MD simulations, as described below. It is important to note that this computational framework has been shown to produce conductance values that are in very good agreement with experimental measurements35.

**Free-energy profiles for K+ and Cl- permeation –** The Adaptive-Biasing-Force (ABF) method36 was used to calculate the potential-of-mean-force that reflects the translocation of either K+ and Cl+ along the lumen of the c-ring, or , for each of the simulation systems described above. The lumen of the c-ring was filled with water (at bulk-like density) throughout these calculations. (It was not required to impose this condition, as this state is metastable and sufficiently long-lived to observe tens of permeation events). The reaction coordinate was the *Z*-coordinate of one K+ or one Cl- ion, defined relatively to the *Z*-coordinate of the center-of-mass of a ring of Cα atoms in the protein (from A22 in the c10-ring, and from A18 in the c13-ring). The permeating ion was confined to a cylindrical volume (through flat-bottom restraining potentials) whose center and axis coincide with the center and axis of the lumen (defined by two additional rings of Cα atoms), and whose radius is slightly larger, namely 8 Å for the c10-ring, and 14 Å for the c13-ring. The length of the cylinder along the *Z*-coordinate was 100 Å, therefore projecting into the bulk solution on both sides of the membrane.

For each ion type and system, two ABF simulations of 300 ns each were carried out, with the permeant ion starting on opposite sides of the membrane; in the course of each of these trajectories, the ions traverse the lumen multiple times, in both directions. The range in the *Z*-coordinate to be sampled was divided up in 0.2-Å bins, and 1000 unbiased samples were collected for each bin before the estimated biasing force was applied and updated. A third simulation was then conducted similarly for 100 ns to combine and refine the bias potentials calculated from the first two simulations. The free-energy profiles shown in **Fig. 1** reflect the outcome of these final calculations, while the error bands reflect the differences between the two initial profiles (averaged over the length of the profile).

**Diffusion profiles for K+ and Cl- across the lumen –** To calculate the diffusion coefficient of the permeant ion as a function of its position along the c-ring lumen, or , 51 independent simulations of 10 ns each were carried out for each system. In each of these simulations, the permeant ion was confined (through flat-bottom restraining potentials) in a disk of 2 Å in length (and radius as specified above), whose center varies in the Z-direction from -50 Å to 50 Å. The starting configurations for each of these simulations were obtained from the ABF trajectories. The position-dependent diffusion coefficient was calculated *a posteriori* from the expression35:

where the numerator is the variance of the *Z*-coordinate of the ion (relative to the protein), i.e. , and is the characteristic time of its autocorrelation function, i.e.:

where , and *t* is the so-called lag time. In our simulations, the values of are all well converged for *t* > 80-90 ps. The diffusion profiles shown in **Fig. 1** are (cubic-spline) interpolation of the 51 values of obtained using this approach; the error bars reflect the differences between values obtained from different halves of the simulations.

**Density-biased multiple-walker Metadynamics –** The Metadynamics method was used to induce the wetting and de-wetting of the lumen of the c10 and c13-rings reversibly, and to calculate the associated free-energy changes. The collective variable biased in these simulations is the water density in the volume of the lumen. Following Feig and co-workers, we express this density as37:

where is a weight function of the Cartesian coordinates of a given atom *i* at a given time, whose value depends on whether that atom is inside () or outside () the volume *V* considered. Given the geometry of the lumen of the c-rings, we used a weight function that defines a cylinder of radius , whose axis and center coincide with the those of the lumen, and which extends from to . Importantly, the boundaries of this cylinder are not abrupt, but rather smoothened over an interval and around and (**Fig. 2 – Supp. 1A**). More specifically:

where (** denotes *z*or *r* and *S* denotes *R* or *Z*) is a switching function whose value varies smoothly from 1 to 0 in the intervals , constructed so that its derivative is 0 at the outer boundaries of the cylinder (**Fig. 2 – Supp. 1B**):

Lastly, the effective volume *V* of this cylinder is:

As in standard Metadynamics, a time-dependent biasing potential *U*b acting on the collective-coordinate was adaptively constructed throughout the simulation; at convergence, the free-energy profile is the negative of this potential. At a given time, the forces acting on a given atom *i* due to this biasing potential can be derived using the chain rule, i.e.:

where , and are the coordinates of atom *i*. Note that our definition of the weight function is such that is non-zero only if atom *i* is found within the smooth boundaries of the cylinder, i.e. in the region where the switching functions and are active.

The collective variable defined above is a function of all the atoms considered in the density calculation. In our case, these atoms are all the water oxygen atoms in the simulation system, which in principle makes the calculation of the density collective variable and related biasing forces prohibitively slow. Because at a given time the value of the weight function (and associated forces) is non-zero only for the atoms found inside the target volume, to speed up the calculations we introduced a ‘neighbor list’ search, whereby a list of water molecules within and in the vicinity of that volume is created and updated regularly, but not at every simulation step (**Fig. 2 – Supp. 1B**). In addition, we note that unlike earlier work37, our implementation defines in reference to the protein coordinates, i.e. not in absolute Cartesian space, and therefore the tumbling and diffusion of the ring in the membrane is unrestricted during the simulations.

The geometric definitions of the target volumes considered for quantification of the free-energies of hydration of the c10 and c13-ring lumens are indicated in **Fig. 2 – Supp. 1CD**. The Metadynamics simulations were carried out using 8 concurrent replicas, or walkers, which update and share a common bias potential but sample different configurations. The biasing potential applied to the density variable consisted of a series of Gaussian functions of width 0.0005 Å-3, added in 4-ps intervals. The height of the Gaussians was gradually diminished throughout the simulations, ultimately reaching a value of 0.0035 kcal/mol; the free-energy profiles were obtained by time-averaging the biasing potential after this time-point38. The total simulation times per walker were 140 ns for the c10-ring (using the last 50 ns for analysis), and 120 ns for the c13-ring (using the last 80 ns for analysis). The ‘neighbor list’ search comprised a region that is 4 Å larger than the target volume in all directions, and the list of atoms therein was updated every 200 simulation steps, resulting in a ~1000-fold speed-up of the calculations.

**FIGURES LEGENDS**

**Figure 1. Evaluation of the ion-conducting properties of the c-ring lumen, assuming a hydrated state. (A)** Cross-section of the c10-ring of the mitochondrial ATP synthase from *S. cerevisiae*. The surface of the protein is colored as follows: Lys and Arg, blue; Asp and Glu, red; other polar residues (and protonated Glu), green; other residues, grey. The ring is oriented such that the interface with the F1 domain, inside the mitochondrial matrix, is up. **(B)** Molecular simulation system employed to study the properties of the c10-ring, shown again in cross-section (blue). The ring is embedded in a model phospholipid bilayer, in 100 mM KCl. K+ and Cl- ions are shown as yellow and green spheres, respectively. Note the c-ring lumen is hydrated. **(C, D)** Potential-of-mean-force (*G*(*z*), PMF) and diffusion profiles for the permeation of either K+ or Cl- across the lumen of the c10-ring (**Methods**). The lack of binding sites for K+ and the 3 kcal/mol free-energy barrier explain the modest K+ conductance; permeation by Cl-, by contrast, is strongly favored electrostatically. **(E, F)** Same as (A, B), for the variant of the c13-ring from *B. pseudofirmus* (**Fig. 1 – Supp. 2BC**)*,* whose lumen is wider than that of the c10-ring. **(G)** Free-energy of selectivity for Cl- and against K+ by the c10-ring lumen, examined with and without electrolyte. The selectivity profile, *G*(*z*), was calculated by subtracting the individual PMF profiles, *G*(*z*), in each case. The marked increase in Cl- selectivity towards the lumen entrance on the matrix side confirms the strong electrostatic influence of a ring of arginine residues. **(H)** Same as (G), for the variant of the c13-ring from *B. pseudofirmus*. Despite its wider lumen, this ring is also markedly anion selective, unlike the MPTP.

**Figure 2. Evaluation of the likelihood of hydration of the c-ring lumen. (A)** Free energy as a function of the water density inside the lumen of the mitochondrial c10-ring, calculated with a variant of the Metadynamics technique (**Methods**), for a simulation system analogous to that shown in **Fig. 1B**. The density value for bulk water (for the CHARMM36 forcefield) is indicated in red. Error bars reflect the differences between two profiles calculated using different halves of all simulation data. **(B)** Snapshots of the c-ring lumen in the two metastable minima detected in the free-energy profile shown in panel (A), i.e. a non-conducting, de-wetted state (left), and a water-filled, putatively-conducting state (right), whose properties are characterized in **Fig. 1**. For clarity, several c-subunits in the c-ring are omitted, as are the lipid bilayer and the solvent outside the lumen. Hydrogen atoms in water (red spheres) are also omitted. Note that in the non-conducting state (left), the two regions at the entrance of the hydrophobic portion of the lumen are hydrated; hence the density value for this state in free-energy profile in panel (A) is not zero. **(C, D)** Same as (A, B), for the variant of the prokaryotic c13-ring from the *B. pseudofirmus* ATP synthase.

**Figure 3. Evidence that lipid molecules block the lumen of the c-ring.** Three independent molecular dynamics simulations were carried out of assembly intermediates of the c10- and c13-rings lacking two of the c-subunits. Initially, the lumen of these c-rings was hydrated at bulk-like density. **(A)** Time-series of the number of non-hydrogen atoms from either water (dashed lines) or lipid molecules (solid lines) inside the lumen of the mitochondrial c-ring. The volume consider for this count is a cylinder of radius 7 Å and height 32 Å, approximately centered in the middle of the membrane. Running averages are shown for each of the three simulation, colored in black, red and blue, respectively, with the raw data shown in the background in grey. The number of atoms equivalent to 4 POPC lipid molecules is indicated, for reference. **(B)** Snapshots of the molecular system, at the beginning (left) and at the end (right) of the simulation. Water molecules (red) are progressively displaced by lipid molecules (shown in yellow) entering the lumen laterally. **(C)** Side-view of the open c-ring at the end of the one of the simulations. The lipid molecules inside the lumen preserve a bilayer arrangement, and adapt to the specific features of the protein surface. **(D, E, F)** Same as (A, B, C), for the variant of the c13-ring from *B. pseudofirmus*. The volume considered in panel (D) is a cylinder of radius 14 Å and height 34 Å.

**SUPPLEMENTARY FIGURE LEGENDS**

**Figure 1 – Supplement 1. Structure of the F1c10-ring subcomplex of the yeast mitochondrial ATP synthase.** The structure shown corresponds to PDB entry 2WPD10. Note that the complete F1Fo enzyme includes additional components both inside and outside the membrane, not present in this structure; these missing components sit peripherally and are believed to act as the ‘stator’ against which the moving parts rotate, and also mediate the dimerization of the enzyme. **(A)** Structure of the c10-ring in the Fo domain, viewed from the plane of the membrane and from the interior of the mitochondrial matrix. Each of the 10 c-subunits consists of two transmembrane helices. The functional proton-binding sites are found on the outermost surface of the ring, roughly halfway across the transmembrane span. **(B)** The F1 domain sits atop the c-ring and includes the catalytic unit (33) and the so-called central stalk (subunits ,  and e), which mechanically couples F1 and Fo. **(C)** Close-up of the interface between the c-ring and the central stalk, highlighting openings that would plausibly permit the downhill permeation of the protons across the c-ring lumen, if this lumen had channel-like characteristics. These openings are considerably larger in ATP synthases with larger c-rings (i.e. with greater number of c-subunits).

**Figure 1 – Supplement 2. Comparison of c-rings.** **(A)** Structure of the c8-ring of the ATP synthase from bovine mitochondria11. Side-chains were modeled onto the low-resolution structure. This c8-ring is thought to be conserved across all vertebrates11. **(B)** Structure of the c13-ring of the ATP synthase from *Bacillus pseudofirmus* OF424, in the wild-type form. **(C)** Double-mutant of the c13-ring, made to resemble the lumen of the c8-ring and c10-rings (**Fig. 1A**).

**Figure 2 – Supplement 1. Graphical definition of the density collective-variable with cylindrical geometry.** See Methods. **(A)** The weight function ξdefines a cylindrical volume with soft boundaries that extend from R-R/2 to R+R/2, and from |Z-Z/2| to |Z+Z/2|. Specifically, the value of ξ inside the blue cylinder is 1, and is 0 outside the red cylinder. In between the two cylinders, ξswitches smoothly from 1 to 0 (panel B). A larger cylinder, shown in grey, is used to define and update a list of particles that neighbor the volume during the simulation. **(B)** Switching function used to evaluate ξ at the cylinder boundaries. **(C, D)** Specific definition of the cylindrical volume used in our analysis of the probability of lumen hydration in the c10- and c13-rings. As mentioned, this volume is defined relative to the protein coordinates, and not in absolute Cartesian space; therefore, the volume can change position and orientation during the simulation as the protein tumbles and diffuses within the membrane. To achieve this, 3 sets of C atoms in regions of the protein with minimal structural variability (orange and cyan spheres) are used to define 3 centers-of-mass. The center of the cylindrical volume is made to coincide and follow the protein center in the middle (orange), while the vector that connects the two distal centers (cyan) defines the cylinder axis.

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