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Predicted Structures of the Proton-Bound Membrane-Embedded Rotor Rings of the *Saccharomyces cerevisiae* and *Escherichia coli* ATP Synthases

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Abstract

Recent years have witnessed a renewed interest in the ATP synthase as a drug target against human pathogens. Indeed, clinical, biochemical and structural data indicate that hydrophobic inhibitors targeting the membrane-embedded proton-binding sites of the c-subunit ring could serve as last-resort antibiotics against multi-drug resistant strains. However, because inhibition of the mitochondrial ATP synthase in humans is lethal, it is essential that these inhibitors be not only potent but also highly selective for the bacterial enzyme. To this end, a detailed understanding of the structure of this protein target is arguably instrumental. Here, we use computational methods to predict the atomic structures of the proton-binding sites in two prototypical c-rings: that of the ATP synthase from *Saccharomyces cerevisiae*, which is a model system for mitochondrial enzymes; and that from *Escherichia coli*, which can be pathogenic for humans. Our study reveals the structure of these binding sites loaded with protons and in the context of the membrane; that is, in the state that would mediate the recognition of a potential inhibitor. Both structures reflect a mode of proton coordination unlike those previously observed in other c-ring structures, whether experimental or modeled.

TOC image

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Supporting Information

Four figures to provide additional background and methodological details.

Introduction

ATP synthases sustain most of the ATP supply required by the cell. To carry out this essential function, these membrane enzymes have evolved a unique mechanism to harness the free energy that results from light harvesting or the metabolism of cellular nutrients, which is then stored as a transmembrane gradient of protons or Na^+ ions^{1–2}. A membrane-embedded component known as the c-ring is key for this energy transduction mechanism. Owing to its structural symmetry, the c-ring has an inherent ability to rotate around its axis relative to the rest of the enzyme, and in particular against an adjacent static element, referred to as subunit-a (Fig. S1). The c-ring also harbors a series of ion-binding sites along its outer circumference, approximately halfway across the membrane. Remarkably, an acidic side-chain is the only feature that is strictly conserved in these binding sites, whose H^+/Na^+ selectivity can vary by up to 10 orders of magnitude, consistent with the diverse metabolic environments in which ATP synthases must operate^{3–5}. It is believed that as the c-ring rotates stochastically and these binding sites enter the interface with subunit-a, they become sequentially exposed to two non-contiguous aqueous pathways, each of which leads to one side of the membrane^{6–9} (Fig. S2). Specifically, in a given snapshot of this mechanism one of the c-ring binding sites would face the channel leading to the interior of the cell or organelle, while the adjacent site, counterclockwise, would be exposed to the exterior. H^+ or Na^+ would be able to enter or exit these channels and thus bind to or be released from the two sites in the c-ring; however, a crucial, strictly conserved arginine on the surface of subunit-a would ensure that no ions hop directly from one of these binding sites to the other^{2, 10–12}. Altogether, these built-in structural features necessarily imply a rotary mechanism of ion transport, whose directionality would be dictated only by the imbalance in the rates of ion binding from either side of the membrane; i.e., by the transmembrane electrochemical gradient.

In its simplest form, the c-ring is an assembly of identical copies of so-called subunit-c, which in most cases consists of two transmembrane α -helices, with a hairpin-like structure^{10–18} (Fig. S3A). In the assembled c-ring, the C-terminal helices form the outermost surface, facing the lipid membrane, while the N-terminal helices form a pore that is most likely plugged by a few lipid molecules^{19–21}. The numbers of c-subunits forming the ring varies across species, but it is believed to be unique for a given organism²². Regardless of the size of the ring, the ion-binding sites are consistently found in between two adjacent outer helices, although side-chains from the inner helices are also implicated^{10–13, 16, 18, 23–24} (Fig. S3B). The distance between adjacent outer helices and between adjacent inner helices must be therefore approximately constant, regardless of the size of the c-ring. Instead, it is the degree of staggering of outer and inner helices that changes quite significantly to accommodate different number of c-subunits^{5, 22}.

Owing to its essential cellular role, the ATP synthase, and in particular its membrane domain, is an appealing pharmacological target^{25–26}. Most cancer cells have a greater energy requirement than normal cells, and therefore inhibitors of the human mitochondrial ATP synthase might be effective in treating some forms of cancer. Similarly, selective inhibitors of the prokaryotic enzyme could be instrumental against strains of infectious pathogenic bacteria that are multi-drug resistant or metabolically dormant. Unfortunately, little is known

about the factors that control the specificity of known inhibitors of the c-ring, or their mechanism of action, hindering further developments. Oligomycin and its derivatives, for example, are known to be potent inhibitors of the mitochondrial ATP synthase²⁷, and to bind to the outer face of the proton-binding sites in the c-ring²⁸, but appear to act much more weakly on bacterial enzymes. Venturicin, despite being structurally and chemically similar to oligomycin, inhibits both eukaryotic and prokaryotic enzymes, including that of *Escherichia coli*. Bedaquiline, by contrast, is thought to selectively inhibit some bacterial ATP synthases²⁹; indeed, this compound was recently FDA-approved as a last-resort antibiotic against multi-drug resistant *Mycobacterium tuberculosis*³⁰. In clinical trials, however, this drug (Surturo) also resulted in an increased risk of death and altered heart function³¹, both of which might be caused by inhibition of the human mitochondrial ATP synthase.

It seems clear, therefore, that there is a need for novel and/or improved inhibitors that combine the required potency and specificity. Although as mentioned the general features of the c-ring are largely conserved across species, considerable variability also exists in the structure and chemical make-up of the ion-binding sites, and in the fine details of the c-ring architecture. Arguably, therefore, novel pharmacological developments in this area would benefit from rational-design approaches that factor in detailed structural information on the c-ring. Here, we sought to add to the existing pool of structural data by studying the three-dimensional structures of two representative c-rings, namely that of the mitochondrial ATP synthase from *Saccharomyces cerevisiae*, whose c-subunit is 60% sequence-identical to the human protein; and that of the ATP synthase from *Escherichia coli*, a pervasive human pathogen whose antibiotic resistance is rapidly developing. Specifically, we used computational methods to predict the atomic structure of the ion-binding sites in these two c-rings, in the proton-bound state and while exposed to the core of the lipid membrane; that is, in the functional state that recognizes of hydrophobic inhibitors such as oligomycin or bedaquiline, which naturally partition into the membrane. The resulting structural models reveal two distinct modes of proton binding not previously observed in any of the c-ring structures that have been experimentally determined thus far.

Theoretical Methods

Homology modeling of the *E. coli* c-ring

A homology model of the c₁₀ ring of *E. coli* in the proton-loaded state was obtained using Modeller³², following a customized multiple-template approach. Specifically, two different templates were used concurrently to model the structure of the individual subunit-c. These two single-subunit templates were extracted from two of the available crystal structures of complete c-rings: that of the Na⁺-loaded c₁₁ ring from *Ilyobacter tartaricus* (22% sequence identity, PDB entry 2WGM, 2.35-Å resolution²¹) and that of the H⁺-loaded c₁₃ ring from *Bacillus pseudofirmus* OF4 (35% sequence identity, PDB entry 2X2V, 2.5-Å resolution^{15, 24}). As a global template of the oligomeric assembly, we used instead the available crystal structure of the c₁₀ ring of *S. cerevisiae* (20% sequence identity, PDB entry 3U2F, 2.0-Å resolution¹¹), which reflects the apo, open conformation of the H⁺-binding sites.

To generate a high-confidence alignment of the *E. coli* c-subunit sequence and those of the selected templates, we first carried out a PSI-BLAST search on the NCBI database for each sequence separately. The sequences retrieved were clustered by sequence identity using a cutoff value of 80%, and representative sequences of each cluster were then aligned with those of *E. coli*, *S. cerevisiae*, *I. tartaricus* and *B. pseudofirmus* OF4, using T-Coffee³³. The resulting alignment of the latter four sequences, shown in Fig. S4A, was then verified by comparing the predicted secondary structure of the *E. coli* target c-subunit, based on PSIPRED³⁴ with the actual secondary structure of each of the templates, as determined by DSSP³⁵.

In the conventional multiple-template approach of Modeller, the ‘initial’ model of the target (to be subsequently optimized) is first generated by averaging the coordinates of the matching atoms in all template structures irrespective of their homology (missing coordinates are generated based on ideal geometries). We have previously shown, in a different context, that an alternative approach can lead to better predictions³⁶. Specifically, for each position in the target sequence, only the template(s) that is most suitable, according to a BLOSUM62 substitution-matrix score, is considered when constructing the initial seed model. As demonstrated in Fig. 1, this multiple-template method also leads to better predictions in the case of the c-subunit. In practice, this customization requires constructing a second multiple-sequence alignment, where gaps are added to indicate which template(s) are to be neglected for each residue position in the target sequence; the alignment used to generate the initial model of the *E. coli* c-subunit is shown in Fig. S4B. Using this ‘initial model’ as starting point, we then followed the conventional simulated-annealing optimization method available in Modeller to generate an ensemble of 2,000 models, and ranked these according to their DOPE³⁷ and GA341³⁸ scores; the top-ranking c-subunit model was then selected.

To construct a model of the c₁₀ ring, the top-ranking c-subunit model was replicated 10 times and each replica was overlaid onto each of the c-subunits in the crystal structure of the c₁₀ ring of *S. cerevisiae*, using MAMMOTH³⁹. This assembly was then used as the seed model for a second round of simulated-annealing calculations, again carried out with Modeller. Specifically, we generated an ensemble of 500 models of the complete c-ring, from which the top-ranking model, based again on the DOPE and GA341 scores, was selected.

Refinement of the *E. coli* c-ring model

Conventional and Free-Energy Perturbation molecular dynamics (MD) simulations were employed to refine the model of the *E. coli* c₁₀ ring, and specifically the structure of the H⁺-binding sites. All simulations were carried out with NAMD⁴⁰ using the CHARMM27/CMAP force-field for proteins and lipids^{41–42}, at constant pressure (1 atm) and temperature (298 K), and with periodic boundary conditions in all directions. Selected calculations were also carried out using the CHARMM36 forcefield. Electrostatic interactions were computed with the PME algorithm; van der Waals interatomic interactions were cut off at a distance of 12 Å, using a smooth switching function taking effect at a distance of 10 Å. GRIFFIN⁴³ was used to embed the c-ring in a pre-equilibrated, hydrated palmitoyl-oleoyl-phosphatidyl-

choline (POPC) membrane; thereafter, the surface area of the membrane was kept constant ($\sim 69 \text{ \AA}^2$ per lipid). GRIFFIN is a simulation-based toolkit that creates a cavity in the interior of an existing lipid membrane, whose dimensions and shape correspond exactly to those of the protein of interest. This method results in a minimal perturbation of the bilayer lipid density, and optimizes polar and non-polar interactions at the protein-lipid and protein-solvent interfaces. The resulting membrane consisted of 239 lipids, 4 of which plug the interior of the c-ring (Fig. 2A). The simulation system amounts to ca. 96,000 atoms, enclosed in an orthorhombic box of approximately $100 \times 100 \times 90 \text{ \AA}$ (Fig. 2A). Selected calculations were also carried out using a palmitoyl-oleoyl-glycero-phosphoethanolamine (POPE) membrane.

The refinement procedure comprised a series of MD simulations adding up to ca. 660 ns in total. In the first ~ 200 ns, the initial conformation of each of the c-subunits was maintained through individual restraints acting on the root-mean-square-deviation (RMSD) of the $\text{C}\alpha$ trace; at this stage, however, the c-subunits were entirely free to rearrange relative to each other. The geometry of each of the binding sites was only loosely preserved in this first simulation, through a series of interatomic distance restraints; by analogy with the c_{13} ring from *Bacillus pseudofirmus* OF4 (one of the templates), a water molecule had been tentatively included in each of the H^+ -binding sites, bridging several interactions between the protonated carboxyl group of Asp61 and the adjacent outer helix, namely with the backbone oxygen atoms of Leu59 and Phe60 and the backbone nitrogen atom of Ile63. Four distance restraints, in the form of a harmonic potential flat-bottomed up to $3.4 \text{ \AA}$, were therefore used in each binding site to maintain this hydrogen-bond network. An identical ~ 100 ns simulation was carried out thereafter, except that this distance restraint was tightened gradually to $3.0 \text{ \AA}$. The abovementioned RMSD restraints were then removed, and in the next ~ 100 ns the c-subunits were allowed adjust their internal conformation freely; however, three series of center-of-mass restraints acting on c-subunit pairs (including $\text{C}\alpha$ atoms of Gln52 to Val56; Leu59 to Ile63; and Ile66 to Leu70) were applied, to loosely preserve the 10-fold symmetry and compactness of the assembly. In the subsequent ~ 50 ns, all restraints were released except those maintaining the binding site geometry. Finally, a completely unrestrained MD simulation was carried out for ~ 50 ns. In this simulation we observed the spontaneous binding of a second water molecule into several of the H^+ -binding sites, resulting in greater stability of the hydrogen-bonding networks therein. We therefore modeled a second water molecule in all binding sites and equilibrated this configuration for 160 ns.

To conclusively ascertain whether two water molecules reside in each of the H^+ -binding sites of the *E. coli* c-ring, we determined their standard binding free energy, using a staged alchemical-perturbation approach. Specifically, we first computed the free-energy change that results from gradually suppressing all interactions between the two water molecules, referred to as WAT1 and WAT2, and the rest of the molecular system, and with each other. We denote this free-energy change as G_{site} . These calculations were carried out with the FEP module of NAMD, forward and backward, in 32 intermediate steps or windows; each window comprised 1.2 ns of FEP-MD simulation (including 0.2 ns of initial equilibration). To facilitate the calculation, a set of distance restraints were used to ensure the two water molecules remain within the binding site even after being decoupled; these restraints, in the

form of a harmonic potential flat-bottomed up to 3.3 Å, were deliberately designed so as to not alter the geometry of the binding site in the coupled state. (Specifically the distances restrained were WAT1:O-WAT2:O, WAT2:O-Asp61:O₈₂, WAT1:O-Leu59':O, WAT1:O-Val60':O, WAT1:O-Ile63':N and WAT2:O-Leu59:O.) The volume explored by each water molecule in the decoupled state is therefore finite; we define these volumes as V_1 and V_2 .

The standard free-energy of binding of the water pair, ΔG^0 , can be derived from these quantities through the expression⁴⁴:

$$\Delta G^0 = \Delta G_{\text{site}} - 2 \times \Delta G_{\text{bulk}} - k_B T \log [V_1/V_o] - k_B T \log [V_2/V_o]$$

where ΔG_{bulk} denotes the dehydration free-energy of a water molecule (6.5 kcal/mol), T is the temperature and k_B the Boltzmann constant. The last two terms correct for the fact that the volume in which the water molecules are confined as they become decoupled is significantly smaller than the standard volume, i.e. $V^0 = 1661 \text{ Å}^3$ (1 M), which translates into an entropic free-energy penalty. To determine V_1 and V_2 , we calculated a three-dimensional density map for each of the oxygen atoms from the FEP windows in which WAT1 and WAT2 are totally decoupled from the system (after removing roto-translations of the c-ring), and then estimated the volume encompassed by each map.

The effective dissociation constant K_d of the water pair can be deduced from the standard binding free energy,

$$K_d = \exp [\Delta G^0 / k_B T]$$

and compared with the bulk-water concentration, $C_W = 55.5 \text{ M}$, to determine the actual probability of occupancy of the c-ring binding sites, i.e.:

$$P_o = [1 + K_d / C_W]^{-1}$$

Structure of the proton-locked binding-sites of the *S. cerevisiae* c-ring

MD simulations were also used to determine the structure of the *S. cerevisiae* c-ring in the proton-bound state, in a phospholipid membrane. These simulations were prepared and carried out similarly to those described above. The starting point of these calculations, however, was not a homology model but the abovementioned crystal structure of this same c-ring, in the putatively proton-free state (PDB entry 3U2F¹¹). This structure was obtained by crystallizing the protein in an amphipathic organic solvent, rather than a conventional detergent/lipid-based buffer. Simulations of the c-ring in this organic solvent were also carried out as control. Specifically, the organic solvent consists of 70% (v/v) 2-methyl-2,4-pentandiol (MPD), 30% water (with 300 mM NaCl), enclosed in a orthorhombic box of approximately $84 \times 84 \times 81 \text{ Å}$ (Fig. 2B). This unusual simulation system was prepared as described previously¹¹; briefly, MPD molecules were initially distributed uniformly across the simulation system, and a series of simulations (approx. 100 ns in total) in which the

crystal structure of the c-ring was restrained were carried out to allow the solvent to partition freely, after which the restraints on the protein structure were gradually released.

Results and Discussion

Predicted structure of *E. coli* c-ring features novel proton-binding motif

The *E. coli* c-ring is known to consist of 10 identical copies of subunit-c⁴⁵; therefore, among available experimental structures, the most suitable template for homology modeling would appear to be the c₁₀ ring of the mitochondrial ATP synthase from *Saccharomyces cerevisiae*¹¹. However, sequence analysis shows that if the c-subunit is considered individually, two bacterial homologues of known atomic structure are better templates, namely the c-subunits from *Ilyobacter tartaricus*²¹ and from *Bacillus pseudofirmus* OF4^{15, 24} (Fig. S4A). Sequence-identity aside, these two c-ring structures are good templates also because they reflect the ion-loaded state of the binding sites, unlike that from *S. cerevisiae*. However, the *I. tartaricus* and *B. pseudofirmus* rings consist of 11 and 13 subunits, and are thus not a suitable reference for the topology of the complete assembly. Therefore, we decided to use all three structures and sequences to model the *E. coli* c-ring, through an approach that makes use of the most suitable template for each position in the sequence (see Methods). For example, as will be discussed below, an important feature of the amino-acid sequence of the *E. coli* c-subunit, shared by that of *B. pseudofirmus* but not the other templates, is a proline residue three positions after the key carboxylic residue known to mediate H⁺ binding (Asp61 in *E. coli*, Glu54 in *B. pseudofirmus*) (Fig. S3B). Therefore, to model that fragment of the protein, the other templates are entirely neglected.

The resulting homology model was subsequently refined via all-atom molecular dynamics simulations in a hydrated phospholipid membrane (Fig. 2A), using a staged protocol whereby global and local structural restraints initially imposed on the protein are gradually weakened, and ultimately removed, in a continuous trajectory of 660 ns in total (see Methods). Fig. 3 shows the overall structure of this c₁₀ ring model, averaged over the last 60 ns of unrestrained simulation. As in other c-rings of small stoichiometry, the outer helices in the c-subunits are not arranged radially relative to the inner helices; instead, they are noticeably staggered (Fig. 3A). This arrangement maximizes the number of contacts between outer and inner helices, and the formation of an ion-binding pocket in between adjacent c-subunits, irrespective of the curvature of the ring.

As expected the H⁺ binding sites are halfway across the transmembrane region, on the outer face of the ring (Fig. 3B). These H⁺ binding sites are primarily hydrophobic (Ala24, Met57, Leu59, Val60, Ala62, Ile63, Met65), aside from the carboxylic side-chain that becomes protonated (here, Asp61). These hydrophobic groups explain why the ATP synthase from *E. coli* is driven by H⁺ only, as they most certainly confer the binding sites with a very strong selectivity against Na⁺, which is several orders of magnitude more abundant than H⁺ under physiological conditions⁵. In addition, the bound proton is structurally 'locked' in a hydrogen-bond in which Asp61 serves as the donor. This is a recurrent pattern in known atomic structures of H⁺-selective rings; for example, Glu61 in the c₁₄ ring from spinach chloroplast (Fig. S3A) interacts directly across the binding site with the backbone carbonyl of Phe59 (equivalent to Leu59 in *E. coli*) in the adjacent c-subunit^{14, 23}; a variation is seen in

the c₁₃ ring *B. pseudofirmus* OF4 (Fig. S3B), where a water molecule bridges the analogous interaction for protonated Glu54^{15, 24}. As mentioned, in the *E. coli* c₁₀ ring the proton-accepting side-chain is not a glutamate, but an aspartate (Asp61). The fact that the aspartate side-chain is shorter than that of glutamate, combined with the larger curvature of this c₁₀ ring, implies that none of the H⁺-binding motifs thus far observed in atomic structures of c-rings is geometrically feasible.

Instead, our simulations indicate that in the *E. coli* c₁₀ ring two water molecules bridge the inter-subunit interaction that is seen in other c-rings between the protonated carboxylic side-chain and the adjacent outer helix (Fig. 3C). More precisely, one of the water molecules is inserted in a kink in the backbone of the outer helix, induced by the abovementioned Pro64, and is coordinated by the carbonyl and amino dipoles that become exposed therein (as mentioned, this motif is identical to that observed in the *B. pseudofirmus* OF4 c₁₃ ring). The second water molecule, not seen in previous c-rings, donates a hydrogen-bond to the first water, and is also the required hydrogen-bond acceptor for protonated Asp61 (Fig. 3C). This result can be reproduced with different force fields and membrane compositions (Fig. 3D–E). The calculated standard binding free energy of this water pair, calculated through Free-Energy Perturbation simulations (see Methods) further substantiates this prediction. Specifically, we obtained $\Delta G^\circ = -1.65$ kcal/mol, which corresponds to a dissociation constant K_d of 62 mM, and therefore a probability of occupancy of 0.999, consistent with the findings of the conventional MD simulations.

Proposed structure of *E. coli* c-ring is drastically divergent from existing NMR model

It is important to note that the structure of the *E. coli* c-ring presented here is markedly different from that originally proposed (Fig. 4, PDB 1C17), based on solution-NMR data obtained for individual c-subunits solubilized in an organic solvent (i.e. neither in the context of a membrane, or the rest of the oligomer)⁴⁶. Aside from its erroneous c-subunit stoichiometry (12 instead of 10) (Fig. 4A–B), that earlier structural model shows the outer helices projecting radially from the center of the ring (Fig. 4B), and thus the only inter-subunit interactions are formed by the inner helices (Fig. 4C). In addition, the outer helices are perfectly straight and rotated around their axis by about one-half of a helix-turn, relative to our model (Fig. 4D). As a result, the geometry and amino-acid composition of the H⁺ binding sites are drastically different (Fig. 4E–F); in that previous structure, Asp61 is not exposed on the outer face of the c-ring, facilitating H⁺ binding to a site between the outer helices of adjacent c-subunits; instead, Asp61 is buried more deeply within the protein, stabilized by hydrogen-bonds with the backbone of the inner helix of the same c-subunit (Fig. 4E–F).

The extent to which our model is an accurate representation of *E. coli* c₁₀ ring can only be conclusively established by an experimental structure of the complete assembly. Nonetheless, it is important to note that both in its global and detailed features, the earlier solution-NMR model is inconsistent with all of the available X-ray structures of intact c-rings, including those not considered here as templates. Recent solid-state NMR measurements for the complete *E. coli* c-ring have also discarded a direct interaction between Asp61 and the inner helix⁴⁷, consistent with our proposal. That residues Ile55,

Ala62, Gly69 and Phe76 are lined up on the outer face of the c-ring, as seen in our model (Fig. 4A), is also in agreement with that solid-state NMR data, which indicates that these four residues are in contact with the lipid membrane⁴⁷; by contrast, all of these residues are buried inside the protein in the solution-NMR c₁₂ model, similarly to Asp61 (Fig. 4B). Unfortunately, all published simulation studies of the mechanism of the membrane domain of the *E. coli* ATP synthase have been based on that earlier, seemingly erroneous model of the c-ring^{48–49}; in future work, therefore, it would be of interest to re-assess the conclusions of those studies in light of the more plausible structural model put forward here.

Concerted side-chain dynamics underlie proton binding to the *S. cerevisiae* c-ring

The known crystal structure of the *S. cerevisiae* c₁₀ ring (PDB 3U2F¹¹) is noticeably different from other known c-ring structures in that the proton-binding sites adopt an ‘open’ conformation, presumably stabilized upon deprotonation, in which the conserved glutamate (Glu59) projects outwards and forms no intra-molecular interactions (Fig. 5A). By contrast, all other c-ring structures show the conserved Glu retracted into the ion-binding site, forming direct or water-mediated interactions with other residues lining the site^{10, 18, 23–24} (Fig. S3). As mentioned, the common denominator of the latter structures is that they reflect the c-ring crystallized in detergent-based buffers, which mimic the hydrophobic core of the lipid membrane^{2, 12}. By contrast, the structure of the *S. cerevisiae* c₁₀ ring was obtained in a partially aqueous organic solvent, primarily consisting of 2-methyl-2, 4-pentandiol (MPD). Elsewhere, we have proposed that it is the hydration of the binding sites and the possibility of hydrogen-bonding interactions between Glu59 and MPD molecules that explain the observation of this open/apo state¹¹. It follows that the atomic structure of the proton-loaded binding sites in the context of the lipid membrane has been unknown, for this or any other mitochondrial c-ring (existing data for the bovine c₈ ring¹⁷ is of insufficient resolution). It is unclear, therefore, how protons are stabilized in those binding sites as they make a revolution within the membrane, and by extension what conformation mediates the recognition of potential hydrophobic inhibitors of the mitochondrial ATP synthase.

To address this question, we simulated the *S. cerevisiae* c-ring embedded in a phospholipid membrane, with Glu59 protonated, and compared the conformational dynamics of each of the proton-binding sites with simulations in which the c-ring was instead immersed in the abovementioned MPD/water buffer, with Glu59 either protonated or deprotonated. Fig. 5 summarizes the results of this comparative analysis. Consistent with what we observe for e.g. the *E. coli* c-ring, the binding sites in the *S. cerevisiae* c-ring primarily adopt a ‘locked’ state if immersed in the lipid bilayer, in which protonated Glu59 retracts into the protein (Fig. 5A–B). This proton-locked state, however, differs from that observed in other H⁺-dependent rings (Fig. S3) in that the stabilizing interaction that bridges Glu59 and the adjacent outer helix (here Leu57) is neither direct (as seen in the chloroplast c₁₄ ring) nor water-mediated (as in the *E. coli* c₁₀ ring above). Instead, this same interaction is mediated by the side-chain of Thr61 (Fig. 5A), whose rotameric dynamics is clearly coupled to that of Glu59 (Fig. 5B). In the open state, i.e. with Glu59 projecting out of the binding site, Thr61 is retracted and locked in position by a hydrogen-bond to Ser58, within the same helix (Fig. 5A–B); when Glu59 retracts to close the binding site, Thr61 isomerizes and provides the hydrogen-bond acceptor required to stabilize the bound proton, while also becoming the

bridge between Glu59 and the backbone carbonyl of Leu57 (Fig. 5A–B). As shown in Fig. 6, these results can be reproduced with different force fields and membrane compositions.

To further verify this result, we carried out several additional simulations of the *S. cerevisiae* ring in which a water molecule was initially modeled into the proton site, bridging the interaction between Glu59 and L57, by analogy to what is observed in the *E. coli* and *B. pseudofirmus* c-rings (Fig. 7A). These simulations were carried out using two types of phospholipid membranes and two different force fields, yielding nearly identical results. That is, most if not all of the 10 proton-binding sites in the c-ring shed the hypothetical water molecule within 100 ns of simulation (Fig. 7B), and Thr61 ultimately becomes the bridging contact for Glu59. We therefore conclude that the configuration of the binding sites in the *S. cerevisiae* c-ring as they carry the bound proton within the membrane is that shown in Fig. 5A (right panel). Interestingly, this previously unseen configuration is also observed when the c-ring is immersed in MPD/water, and Glu59 is protonated, co-existing in a dynamic equilibrium with the open state (Fig. 5B); nevertheless, the most populated state in MPD/water is the open state, particularly if Glu59 is deprotonated, consistent with the crystal structure (Fig. 5B).

Conclusions

We have presented a novel model of the atomic structure of the c₁₀ ring of the *E. coli* ATP synthase, constructed by homology with several of the most recent experimental crystal structures, and refined in a lipid-membrane environment through molecular dynamics simulations and free-energy calculations. Both in its overall architecture and in the chemical structure of the proton-binding sites, our model is drastically different from the existing c₁₂ model originally proposed on the basis of solution-state NMR data for the individual subunit-c⁴⁶, which is also at odds with more recent solid-state NMR measurements obtained for the assembled ring⁴⁷. Given that the most of the published functional and biochemical data for the ATP synthase derives from the *E. coli* enzyme, we expect that the more plausible c-ring structure proposed here will foster new mechanistic insights, particularly through computational approaches; indeed, the seemingly gross inaccuracies of the NMR c₁₂ model call into question the purported realism of existing simulation studies based upon that model⁴⁹.

We also report a prediction for the structure of the ion-binding sites in the c₁₀ ring from *S. cerevisiae* in the proton-locked state, i.e. the state of the binding sites that face the lipid bilayer as the c-ring rotates within the membrane. This prediction is also based on molecular dynamics simulations, not of a homology model, but of a crystal structure of this same ring, obtained in a non-membrane environment and with the binding sites in a proton-free, open form¹¹. These simulations revealed that the gating mechanism of these binding sites involves concerted side-chain isomerizations, and that it is markedly sensitive to the degree of local hydration; this is an interesting finding, as the c-ring binding sites are thought to become exposed to aqueous channels when they enter the interface with an adjacent, static protein in the ATP synthase complex, namely subunit-a.

In known H⁺-dependent c-rings, the protons that bind to the conserved Glu/Asp in the outer helix of each subunit-c are locked by direct or indirect hydrogen-bonds with the backbone of the adjacent outer helix^{10, 18, 23–24}. The two c-ring structures proposed here lock the bound H⁺ in two distinct ways, neither of which had been previously observed in the c-rings of ATP synthases. In *S. cerevisiae*, a side-chain provides a bridging interaction between Glu59 and the backbone of Leu57, on the adjacent outer helix; in *E. coli*, two structural water molecules bridge the exact same interaction between Asp61 and Leu59. Available structural data indicates that compounds that inhibit the ATP synthase by targeting the c-ring binding sites do so because they become integrated in the network of interactions therein. Thus, we anticipate that the c-ring structures reported here, arguably from the two most prototypical ATP synthases, will be of value in future efforts to improve the potency and specificity of inhibitors of this class of essential enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Evaluation of the accuracy of our customized two-template homology-modeling method, for a c-subunit of known structure

To evaluate the accuracy of the methodology used to model the c-subunit of *E. coli*, we modeled the structure of the c-subunit from *Mycobacterium phlei*, which has been determined through X-ray crystallography¹⁸. As templates, we used the c-subunits from *Saccharomyces cerevisiae*¹¹ (29% sequence identity, 2.0 Å resolution) and from *Spirulina platensis*² (34% sequence identity, 1.9 Å resolution). **(A)** The actual structure of the *M. phlei* c-subunit (marine ribbon) is compared with single-template models based on the c-subunits of either *S. cerevisiae* (yellow) or *Spirulina platensis* (red). The root-mean-square difference (RMSD) between the C α trace of each model and that of the actual structure, excluding the N- and C-termini (gray), is indicated. The values for the complete trace are 5.0 and 5.9 Å for the two single-template models (yellow, red) and 2.6 Å for the two-template model (green). **(B)** The actual structure of the *M. phlei* c-subunit (marine ribbon) is compared with a model based on our customized two-template approach (green), which is clearly the most similar to the correct structure.

Figure 2. Molecular simulation systems

(A) The c-subunit ring of the ATP synthase from *E. coli* (yellow), embedded in a POPC bilayer, and viewed along the mid-plane. The system comprises approximately 100,000 atoms. (B) Interior of the *E. coli* c-ring, plugged by 4 lipid molecules. Six c-subunits are shown in a surface representation, and colored as follows: hydrophobic, gray; acidic, red; basic: blue; polar, green. Two additional c-subunits are shown as helical cartoons (yellow). Water molecules hydrating the POPC head-groups and the protein are also highlighted. (C) The c-subunit ring from the mitochondrial ATP synthase from *S. cerevisiae* (yellow), viewed along the axis of the ring. The ring is immersed in a solution of consisting of 2-methyl-2,4-pentanediol (MPD, marine) in water (70% v/v). (D) Close-up of the *S. cerevisiae* c-ring in the MPD/water buffer, in the same format as that used in panel (B). An MPD molecule is shown in the inset. Non-polar hydrogen atoms and electrolyte and counter-ions are omitted, for clarity.

Figure 3. Predicted structure of the H^+ -driven c-ring of the *E. coli* ATP synthase

(A) Overall architecture of the c-ring, which consists of 10 c-subunits, viewed from the cytoplasmic site along the ring axis. Dashed black lines approximately connect the center of the inner (or N-terminal) and outer (or C-terminal) helices in each c-subunit, to indicate their staggered (as opposed to radial) configuration. The side-chain of Asp61 is highlighted (yellow, red), to indicate the location of the 10 proton-binding sites. Two water molecules (blue density-map) are also bound to these sites. (B) View of the c-ring along the bilayer plane, in cross-section, with the cytoplasmic or electro-negative (N) side above, and the periplasmic or electro-positive (P) side below. Dashed black lines approximately indicate the transmembrane span. (C) Close-up of the structure of the H^+ binding sites, highlighting Asp61, the two bound water molecules (W), and the pattern of hydrogen-bonds that lock the proton in place, in addition to the surrounding hydrophobic side-chains. Non-polar hydrogen atoms are omitted for clarity. (D, E) Comparison of the calculated water density maps shown in panel (A) with independent simulations carried out with the CHARMM36 forcefield, for both a POPC and a POPE lipid bilayer. The results are nearly identical to those obtained with CHARMM27 and POPC.

Figure 4. Comparison of the existing and newly proposed structural models of the c-ring of the *E. coli* ATP synthase

(A, B) Our proposed structure of the *E. coli* c_{10} ring (marine), and that previously published (orange, PDB 1C17)⁴⁶, respectively. The c-rings are viewed from the cytoplasmic side. Dashed black lines approximately connect the centers of the inner and outer helices of two opposing c-subunits, relative to the center of the ring. Residues revealed to be exposed to the lipid membrane by solid-state NMR experiments⁴⁷ are indicated by their $C\alpha$ atoms. Note that all these residues are on the outer face of the ring in our structure, but are buried between the outer and inner helices in the previous model. (C) Close-up of the interior of the central pore, illustrating the different packing of the inner helices in both models (only the c' subunits are optimally fitted), owing to their differing stoichiometry. (D) Comparison of the structure of the individual c-subunits in each model (only residues 2–70 are shown); the inner helices are overlaid optimally. Note the pronounced difference in the position, conformation and orientation of the outer helix. The side-chain of Asp61 (indicated by the $C\alpha$ atom) projects in opposing directions. (E, F) Close-up view of three adjacent c-subunits, in the region of the H^+ -binding sites, highlighting several side-chains on the outer helix; similarly to Asp61, all these side-chains have opposing orientations. Hydrogen atoms are omitted for clarity.

Figure 5. Gating mechanism of the H^+ -binding sites in the *S. cerevisiae* c-ring

(A) Left, open-state conformation of the H^+ -binding site in the available crystal structure of the *S. cerevisiae* c-ring, obtained using an amphipathic solvent (MPD/water) as the crystallization buffer¹¹. The structure is also likely to reflect Glu59 in equilibrium between deprotonated (not shown) and protonated states (shown). Either way, Glu59 projects out of the binding site, while Thr61, in the adjacent outer helix is retracted and forms a hydrogen-bond with the backbone carbonyl of Ser58. Right, closed-state conformation revealed in the MD simulations reported here (see Methods); Glu59 retracts into the binding pocket, while Thr61 rotates outward, so as to bridge the interaction of Glu59 with Leu57, which, seemingly locks the bound proton. (B) Calculated probability distributions for distances between key sidechains in the *S. cerevisiae* c-ring; the distributions include data for all proton-binding sites. The data is evidence that the dynamics of Glu59 and Thr61 upon H^+ binding (to Glu59) is concerted, whether in context of the membrane or in the amphipathic MPD buffer. The membrane environment, with Glu59 protonated, strongly favors the conformation shown in panel (A), right; by contrast, the MPD/water buffer (Fig. 1B), with Glu59 deprotonated, strongly favors the conformation shown in panel (B), left. If Glu59 is protonated and the ring is immersed in MPD/water, both open and closed forms coexist in equilibrium, albeit the open state is favored.

Figure 6. Comparison of results obtained with CHARMM27 and CHARMM36 forcefields, in POPC or POPE membranes

(A) Calculated probability distributions for the distance between Glu59 and Ala22 in the *S. cerevisiae* c-ring versus that between Glu59 and Thr61. The distributions include data for all binding sites in the ring. Irrespective of the forcefield used or membrane composition, the data shows that in the context of the membrane, the closed-state of the binding sites (indicated by Glu59-Ala22 distances in the 5.5 to 7.5 Å range) is stabilized not by bound water, but by the side-chain of Thr61, which rotates away from its interaction partner in the open state (Ser58), to form direct hydrogen-bonding interactions with protonated Glu59 and Leu57; as shown in the figure, Thr61 occasionally returns to the open-like rotamer, but the configuration in which Thr61 locks Glu59 in the closed-state is by far the most probable. (B) Geometry of this proton-locked state, in representative snapshots of the CHARMM27 and CHARMM36 simulations with the *S. cerevisiae* ring embedded in POPC or POPE bilayers.

Figure 7. Gating mechanism of the H⁺-binding site of the *S. cerevisiae* c-ring

(A) Hypothetical alternative configuration of the H⁺ binding site in which a water molecule (W) was modeled so as to bridge the interaction between Glu59 and Leu75/Ser58, similarly to what is seen in the c-rings of *B. pseudofirmus* OF4 (Fig. S3) and *E. coli* (Fig. 3C). (B) The plausibility of the configuration shown in panel (A) is evaluated through simulations, and compared with an analogous analysis for the proton-binding sites in the *B. pseudofirmus* c₁₃ ring²⁴ and the *E. coli* c₁₀ ring. For the *E. coli* and *S. cerevisiae* c₁₀ rings, independent simulations were carried out with the CHARMM27 and CHARMM36 forcefields, with the ring embedded in either a POPC or POPE lipid bilayer. The plot quantifies the total water occupancy number of all H⁺-binding sites in each c-ring. Irrespective of the simulation conditions, most if not all of 10 binding sites in the *S. cerevisiae* c₁₀ ring shed the hypothesized water molecule, and adopt the conformation shown in Fig. 5A, right panel.