Structure and Flexibility of the C-Ring in the Electromotor of Rotary F_{0}F_{1}-ATPase of Pea Chloroplasts

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Abstract

A ring of 8–15 identical c-subunits is essential for ion-translocation by the rotary electromotor of the ubiquitous F_{0}F_{1}-ATPase. Here we present the crystal structure at 3.4Å resolution of the c-ring from chloroplasts of a higher plant (Pisum sativum), determined using a native preparation. The crystal structure was found to resemble that of an (ancestral) cyanobacterium. Using elastic network modeling to investigate the ring’s eigen-modes, we found five dominant modes of motion that fell into three classes. They revealed the following deformations of the ring: (i) ellipsoidal, (ii) opposite twisting of the luminal circular surface of the ring against the stromal surface, and (iii) kinking of the hairpin-shaped monomers in the middle, resulting in bending/stretching of the ring. Extension of the elastic network analysis to rings of different c-subunit symmetry revealed the same classes of dominant modes as in P. sativum. We suggest the following functional roles for these classes: The first and third classes of modes affect the interaction of the c-ring with its counterparts in F_{0}, namely subunits a and bb'. These modes are more likely to be involved in ion-translocation and torque generation. The second class of deformation, along with deformations of subunits γ and ε, might serve to elastically buffer the torque transmission between F_{0} and F_{1}.

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Introduction

ATP (adenosine tri-phosphate), the general energy currency of the cell, is supplied mainly by F_{0}F_{1}-ATPase (ATP synthase). This enzyme is composed of two rotary machines, F_{0} and F_{1}, which are coupled by a central rotor and a peripheral stator. F_{0} translocates ions (mostly protons) and generates torque at the expense of the ion-motive force, and F_{1} synthesizes ATP at the expense of the ions (mostly protons) and generates torque at the expense of the ATPase in chloroplasts, CFoCF_{1} [1,10], is the subject of this work. The c-ring of the enzyme’s CFo portion consists of 14 copies of subunit c [11,12], and is embedded in the membrane. The ring is attached to a complex consisting of subunit a and a dimer comprising two b-type subunits; the dimer connects F_{a} with F_{1} (in chloroplasts this dimer is a bb' heterodimer). Despite being bound to abb', the c-ring is able to rotate relative to these subunits. Subunit a hosts an essential arginine residue, which faces the essential glutamic acid residue situated at the center of a proximate hairpin-shaped monomer of c-subunit. Subunit a also contains two proton half-channels connecting the acidic residue on the c-subunit with the lumen and the stroma phases. Brownian fluctuations, together with the ion-driven rotation of the c-ring relative to the two half-channels on subunit a, are responsible for generating the torque associated with rotary proton translocation (see [5,13,14]). The stepped rotary motion of the c-ring relative to the stator has recently been resolved experimentally for the E. coli enzyme [15,16]. The F_{1} portion of the ATP synthase is characterized by a 5-fold stepping rotation; the mismatch between this rotation and the 8-to-15-fold stepping of the F_{0} portion of the ATP synthase is buffered by an elastic power transmission between these motors. For the E. coli enzyme, the stiffness of the stator [17] and a great elastic compliance of the rotor have been determined (see [18,19,20,21], reviewed in [5]). These functional features of the FoF_{1}-ATPase are probably shared by the V-ATPase, which is characterized by similar structures [22].
The previously determined crystal structures of subunit c have provided important insight into the role of the c-ring in the proton translocating mechanism. In a given organism, the c-ring, composed of 8–15 monomers as noted above, encompasses an ion-binding site approximately in the ring’s middle plane [6,12,23,24,25,26,27]. The functional acidic residue (mostly glutamate, in some organisms aspartate) is situated at the outward-facing helix of the hairpin. It is stabilized by hydrogen bonds with neighboring residues. The dynamics of the ion-binding site have been simulated by molecular dynamics (MD), revealing structural transitions at the nanosecond time scale upon glutamate deprotonation/protonation [28,29]. However, MD simulations have been restricted to a narrow time window of 10–100 ns and have not revealed large-scale motion in micro- to milliseconds as relevant in the present case: When F0 is decoupled from the F1-portion, its unitary conductance is approximately 10 IS (for chloroplasts see [30], for Rhodobacter capsulatus [31], and for E. coli [32]), which implies about 1,000 rounds per second of the c-ring (at 200 mV driving force).

Simple techniques for coarse-grained simulations of protein dynamics, e.g., elastic network analysis, take just the Cα-atom of each residue into account, and the solvent is usually neglected. Such an approach does not require extensive computational power and may, in principle, cope with micro- to millisecond events [33]. This restricted approach does not account for the over-damped character of protein dynamics. More advanced techniques have included solvent-protein interactions, damping and thermal properties (for an application to adenylate kinase see [34]); however, simulations are still carried out on a relative and not on a real time scale.

Herein we applied normal mode analysis (without damping) to the c-ring of F0. The purpose was to identify the basic deformation modes of the c-ring, and to investigate whether and how the dominant elastic modes might contribute to the function of the ring in this rotary ion-translocator. The method relies on the position of the Cα-atoms embedded in an elastic network (in vacuo) with a single force constant between neighboring nodes. Orthogonal eigen-modes of motion are computed [33]. For various proteins, including membrane channels and transporters, the slowest (i.e., global or cooperative) modes of motion have been shown to represent functionally significant movements [33,35]. The isotropic Gaussian Network Model (GNM) has been used to assess residue fluctuations and inter-residue dynamical correlations [36,37], and the Anisotropic Network Model (ANM) has been used to identify motion directionality in three dimensions (3D) [38].

We isolated the c-ring of the F0F1-ATPase of the green pea (Pisum sativum) from a native preparation, crystallized it, and determined its homo-tetradecameric structure at a resolution of 3.4 Å. The elastic eigen-modes of the ring structure were determined by GNM and ANM, and the same analysis was applied to c-rings from other FoF1-ATPases containing 8 to 15 copies of subunit c, respectively, and to a ring of a V-ATPase. We discuss the roles of the dominant eigen-modes in rotary proton translocation, in the stepped torque generation, and in the elastic buffering of the stepped rotation for smooth torque transmission into F1.

**Materials and Methods**

**CFoCF1 and c-ring purification**

Thylakoid membranes were purified from ~800 g P. sativum var. Alaska young leaves as in [39]. CFoCF1 complexes were released from the membrane using 0.4% n-dodecyl-β-D-maltoside (DDM, Glycon, Inc.). The preparation was further purified by 2 steps of differential precipitation using PEG 2K as a precipitate using 7% and 9%, respectively. The pellet was dissolved with 20 mM Tricine-Tris (pH 7.4), 0.125 mM dihidroethiol (DTT), and 0.05% DDM, was applied to a 10-40% sucrose gradient containing the same buffer, and was centrifuged using the SW-40 rotor (Beckman Inc.) at 37,000 rpm for 16 h. Fractions containing CFoCF1 were pooled together and loaded onto an ion exchange column (DEAE-cellulose, DE52, Whatman, Inc., 1.5 × 18 cm) pre-equilibrated with 20 mM Tricine-Tris (pH 7.4), 0.125 mM DTT and 0.05% DDM. The column was washed with 40 ml of the same buffer, and fractions containing CFo dominated with subunit-c were eluted by 50–250 mM NaCl linear gradient in 20 mM Tricine-Tris (pH 7.4), 0.125 mM DTT and 0.05% DDM. These fractions were then pooled together and concentrated to 3–4 mg/ml by 2 steps of differential precipitation using PEG 6K as precipitant. The pellet was suspended in 5 mM Tris pH 8.0 and 0.05% Fos-choline 12.

**Cryystalization and structure determination**

Subunit-c crystals grew in a crystallization buffer containing 100 mM Na-acetate (pH 4.5), 50 mM MgCl₂, 50 mM NaCl, 10 mM ytrrium chloride, and 14–24% PEG 550 monomethyl ether (MME), reaching their maximal size after 5–7 days. Ytrium was added using a commercial additive kit (Hampton Research) as it improved the shape of the crystals. After the crystals were equilibrated and then incubated for 48 hours at 20°C, the quality of the crystals and the subsequent diffraction pattern were improved by adding to the reservoir 40% PEG 550 MME. The crystals were then flash frozen in liquid nitrogen. Data were collected in ID23-1, ESRF, Grenoble and processed by XDS [40]. Due to anisotropic diffraction, ellipsoid truncation of the data was performed using the UCLA MBI Diffraction Anisotropy Server [41]. Resolution limits were 3.7, 3.6 and 3.4 Å along a, b and c axes, respectively. Initial phases were determined by maximum-likelihood molecular replacement as implemented in Phaser [41]. The functional acidic residue (mostly glutamate, in some organisms aspartate) is situated at the ion-binding site approximately in the ring’s middle plane [6,12,23,24,25,26,27]. The functional acidic residue (mostly glutamate, in some organisms aspartate) is situated at the ion-binding site approximately in the ring’s middle plane.

**Sequence, evolutionary conservation and pKa calculations**

Using the sequence of subunit-c from the green pea, we initiated a BLAST search against the UniRef90 database [47], collecting 351 sequences with Evalue <0.0001. The full sequences were then aligned using MAFFT [48] and input into the ConSurf webserver [http://consurf.tau.edu.il, [49]) to compute conservation scores. The conservation profiles were then mapped onto the c-ring structure.

The sequences of the c-rings with available structures were aligned using the 3D-Contact software package, and the alignment process was further guided by the structural data [50]. Slight manual adjustments were performed in order to achieve better compliance with the known sequence anchors, namely the TM1-TM2 loop and glycine-containing motifs.

pKa values of Glu61 were computed using the PROKA server [51]. Figures were prepared using the PyMol molecular viewer (http://www.pymol.org/).
GNM and ANM computations

In GNM and ANM, the structure is viewed as a collection of nodes, derived from the Ca atoms, and springs, connecting the nodes according to a given distance cutoff [36,37,46]. The normal modes of motion are determined by the protein's structural architecture, and are ranked according to their 1/eigenvalues from slow to fast, i.e., from the most cooperative modes to local fluctuations. Based on the modes’ contribution to the overall motion, derived from the 1/eigenvalues, we identified five main modes of motion. As observed for other symmetric membrane structures (e.g. [35,32,33,34,55]), some of the modes were degenerative; two types of motion (types I and III, discussed above) were each derived from a combination of two symmetry-related, GNM modes, exhibiting essentially the same frequency. We thus averaged the GNM fluctuations and inter-residue cross-correlations of each pair of modes to receive symmetrical behavior for all subunits. The three types of motion, referred to as types I, II and III, consist of GNM1-2, GNM3 and GNM4-5, respectively. The hinges were derived from the residue fluctuations of each type of motion, denoted as minima, i.e., regions demonstrating significantly low mobility relative to the rest of the residues. To obtain the directions of these structural displacements in 3D space, we employed ANM using the HingeProt [56] and the ANM welservers [57]. The GNM modes were associated to the ANM modes on the basis of inter-residue cross-correlations (Fig. S4). The same analysis was performed for additional c-ring structures, namely, Protein Data Bank (PDB) IDs 2x2v, 2xnd, 2xok, 1yce, 2u2, and 2wei [12,23,24,25,26,58]. Further details are available in the Supporting Text S1.

Results

Overall structure of chloroplast ATP synthase subunit-c

The membrane-embedded domain of chloroplast ATP synthase, including subunit-c, was purified from the native holoenzyme by ion exchange chromatography, and further purified in two precipitation steps with polyethylene glycol (See Materials and Methods). This process, in contrast to previous pre-crystallization processes for c-rings [59,60], was performed without any intense treatment such as harsh detergent or heating. This approach enabled us to crystallize the chloroplast c-ring of a higher plant (P. sativum) from its native environment. The crystal structure of the chloroplast ATP synthase c-ring was determined at 3.4Å resolution with R-work/free of 29% and 32%, respectively, with no outliers in the Ramachandran plot (Table 1).

The crystal lattice revealed one tetradecameric ring in the asymmetric unit, forming crystal contacts with three additional rings. One contact region consisted of loop-to-loop interactions mediated by yttrium ions, as indicated by strong electron densities. The remaining interactions involved the N-termini of three monomers with three additional monomers of two symmetry mates. This interaction was mediated by distinct electron density, which was difficult to interpret (Fig. S1A). Interestingly, another crystal contact was generated in the hydrophobic region located between two rings (Fig. S1B). This inter-space was wide enough to incorporate detergent molecules, which can replace the annular layer of thylakoid membrane lipids or the lipids themselves. Indeed, we observed electron density in this region that resembled a lipid-like structure. The onset of the hydrophobic chain was situated parallel to Phe76, mapped to the luminal boundary of the thylakoid membrane, protruding towards the membrane center (Fig. S1B).

The crystal structure of the c-ring from P. sativum (see Fig. 1) shows a concave barrel shape with a pronounced waist in the middle, exhibiting tetradecameric symmetry [11] consisting of an inner and an outer ring. The structure was 60.5Å long, with outer ring diameters of 60.5Å and 61.5Å at the stroma and lumens sides, respectively (Fig. 1A), whereas the inner ring had a diameter of 35Å at both sides of the membrane (Fig. 1B). The narrowest diameter, 27Å, was mapped to the middle of the ring at Ile22 and Gly23 (Fig. 1A). Each monomer was a hairpin-shaped, 81-residue polypeptide, containing two trans-membrane (TM) helices; TM1 (residues 4–41) composed the inner ring, and TM2 (residues 46–75) formed the outer ring. Both the C- and N-termini were positioned towards the lumens. TM1 and TM2 were connected by a short hydrophilic loop located at the stroma (residues 42–45). TM1 and TM2 were both kinked towards the membrane (with angles of 31° and 20°, respectively), with the kink mapped to the region around Gly23 and Gh61.

According to the hydrophobicity profile [61], residues Lcu54 and Phe76 line the hydrophobic boundaries of the c-ring, surrounded by hydrophilic residues on both ends (Fig. 1C). This indicates that the core membranal region of the c-ring spans 32Å, which potentially correspond to the hydrophobic core width of thylakoid membranes at the ATP synthase location. Additionally, a pronounced hydrophilic patch is apparent at the center of the membrane region, representing the proton-binding site, which is further described below (Fig. 1C). Evolutionary conservation analysis, mapped onto the c-ring structure, was in agreement with the observed structural and functional features. The highly conserved residues (receiving ConSurf grades of 8 or 9 [49]) included the hydrophilic membrane-exposed binding site and surrounding residues, as well as the stroma-facing loop (Fig. 1D).

The latter is implicated in the interaction with the γ-ε globular region, part of the F1 component [6]. Additional conserved positions were detected at helix-helix interactions. Variable positions, i.e., those receiving ConSurf grades of 1 or 2, mapped to the remaining lipid-exposed residues (Fig. 1D), as well as to positions facing the ring interior, a region that probably does not possess a functional or structural role (Fig. 1D).

The proton-binding site retains a chemical coordination similar to that of the alkalophilic cyanobacterium Spirulina platensis (Fig. 2), including the same amino acids: Glu20 and Gh61, located on TM1 and TM2, respectively, of the same monomer, and Phe59 and Tyrr66, which are positioned on TM2 of an adjacent monomer. All four residues were clearly identified in the electron density map (Fig. S2). A network of hydrogen bonds stabilizes the highly conserved Gh61 (Fig. 2).

Gh61 is likely to be protonated in the structure, which was crystallized at pH 4.5, and this notion is supported by a pKa analysis [51]. The Oε2 part of the carboxyl group of Gh61 generates hydrogen bonds with the Nε2 of Glu20 and the carbonyl group of Phe59 (with distances between relevant atoms of 3.1Å and 2.9Å, respectively). The second oxygen group, Oe1, forms a hydrogen bond with the hydroxyl group of Tyrr66 (2.9Å). This chemical coordination represents a locked conformation of the binding site [24].

Normal mode analysis

We analyzed the cooperative movement of the c-ring structure in terms of elastic network models. The motion patterns of residues and the cross-correlations between residues were computed using GNM [36,37]. The directionality of this motion was derived from associated ANM modes [38]. We focused on the five dominant modes, which fell into three classes, referred to as types I, II and III (Fig. 3A). Type I was two-fold degenerate, and the associated deformation of the ring ellipsoidal. Type II was undegenerate and
the deformation torsional, and type III was two-fold degenerate with kinks in the middle.

We carried out the same normal mode analysis for the structures of six other c-rings of FoF$_1$-ATPases, in which the respective numbers of c-subunits varied from eight to fifteen, as well as for the functionally-related ring of a V-ATPase, comprising monomers of four TM helices each [27]. All revealed very similar modes to those of the green pea c-ring, with the same three types of motion (see Fig. S3, Table S1). The similarity of the dominant slow modes among these different enzymes was not very surprising considering their common toroidal topology. Comparable modes have been reported for an unrelated toroid, the nuclear pore complex [62].

In all three types of motion of the *P. sativum* c-ring, the hinging regions, i.e. the least mobile residues in the GNM analysis, were in similar locations. They were clustered at positions 21–26 and 60–64, approximately at the kinked central regions of the two TM helices (Fig. 3B and 3C). Notably, the essential acidic residue of the proton-binding site, Glu61, was part of the hinge region. Gln28 and Phe59, also involved in the ion-binding site [24], resided in close proximity to the hinge. The hinge region was more pronounced, i.e. less mobile, in motion-types II and III, and was less pronounced in type I (Fig. 3B). Although all three types of motion shared the same hinge region, the inter-residue correlations significantly differed (Figs. 4A, 4B, 5, 6A and S4). Typically, GNM and ANM modes are matched according to the residue fluctuations, specifically, the location of the hinges. In this analysis, however, as the hinge locations were essentially the same in all classes of motion, they could not be utilized to correlate the GNM and ANM results. We thus relied on the inter-residue correlations to associate ANM modes to each type of GNM-derived motion (Fig. S4).

### Type I

In this type of motion, the inter-residue correlation separated the c-ring into two oppositely-correlated dynamical elements, divided by a plane perpendicular to the membrane (Figs. 4A and 4B). The associated ANM modes (ANM1–4; Fig. S4 and Table S1) manifested an ellipsoidal deformation, emphasized at the lumen-facing ends in ANM1 and ANM2, and at the stroma-facing loops and helical regions in ANM3 and ANM4 (Figs. 4C, 4D and Movies S1 and S2). In this motion, the ring expands and contracts; opposing monomers approach the center of the c-ring, while the rest of the monomers simultaneously move outwards. This motion changes the overall shape of the ring, transforming its initial round shape into an elliptic one.

### Type II

In this motion, the c-ring structure was divided into two dynamical elements, with a plane separating the helices into lumen- and stroma-facing halves, passing through the hinge region at the membrane center (Figs. 3B and 5A). One dynamical element consisted of the helices’ lumen-facing halves (residues 3–21 and 63–81), oppositely correlated to the second element, which includes the stroma-facing halves and the short loop (residues 24–60) (Fig. 5). Correspondingly, the matched ANM mode (ANM5; Fig. S4 and Table S1) displayed a rotational motion of the two dynamical elements in opposite directions (Fig. 5 and Movie S3), referred to as a twisting motion. The lumen-facing halves rotated clockwise while the stroma-facing halves simultaneously rotated counter clockwise, and vice versa.

### Type III

The inter-residue correlation divided the structure into four main dynamical elements. Each of these four elements consisted of either the lumen- or the stroma-facing halves of approximately five monomers, divided by the hinges at the middle plane of the membrane (Figs. 3B and 6A). Negative dynamical correlation was detected between stroma- and lumen-facing halves of the same five monomers (Fig. 6A). Positive dynamic correlation was observed between stroma-facing halves and lumen-facing halves situated on monomers of opposing sides of the ring (Fig. 6A). The matched ANM modes (ANM6–9; Fig. S4 and Table S1) consisted of a bending and stretching motion, governed by the hinges at the center of the ring (Figs. 3B and 6B, Movie S4). The four dynamical elements described above endured the largest structural displacements during the motion, while the monomers between them mediated the motion. This bending and stretching motion altered the exposure of the hinge region at TM2 towards the membrane.

### Discussion

This work presents a hybrid approach of X-ray crystallography and computational analysis to reveal both fine and gross structural properties of the c-ring of the FoF$_1$-ATPase in higher plant chloroplasts. We discuss the three types of elastic slow motions of the c-ring, in interaction with subunits a and b’.

**Crystal structure of the c$_{14}$-ring of the FoF$_1$-ATPase in the pea**

High-resolution crystal structures of the FoF$_1$-ATPase c-rings of various organisms have been determined [6,12,23,24,25,26]. The ring’s resilience to high temperature and harsh detergents has
facilitated the preparation of 2D-crystals for atomic force microscopy and electron microscopy and of 3D-crystals for X-ray structure analysis. It is an interesting possibility that crystallization under harsh conditions "purifies" certain structural variants. To address this, we used a very mild preparation and crystallization technique, starting from the native holoenzyme.

Figure 1. Architecture of the chloroplast ATP synthase subunit-c. A&B Side (A) and stroma (B) views of the ring, with differently colored monomers. The ring dimensions are marked, and blue arrows indicate the narrowest ring region. C. Side view of the structure in surface presentation, colored according to the hydrophobicity scale below. Left: the membrane boundaries according to the hydrophobicity profile are marked, mapped to Leu54 and Phe76. The hydrophilic residues reside at the ring edges, corresponding to extra-membrane regions, as well as at the membrane center, at the proton-binding site. Right: slab view, displaying the interior of the ring. D. The structure is viewed as in panel C and colored according to evolutionary conservation as calculated by the ConSurf webserver (http://consurf.tau.ac.il, [49]), with cyan-to-maroon indicating variable-to-conserved positions, according to the color bar. Left: the hydrophilic proton-binding site at the membrane center is highly conserved, as are the stroma-facing loops. Right: a slab view reveals that residues lining the interior of the ring are highly variable. Indeed, this region is not expected to possess a functional or structural role.

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order to overcome the common heterogeneity of plant material, *P. sativum* var. Alaska was used under controlled growth conditions. By utilizing a mild approach, we hoped to capture a native form of the c-ring structure, revealing the native protein/lipid-interaction. To the best of our knowledge, the special electron density detected at the crystal contacts between hydrophobic regions of the c-ring is a unique feature, which has not been observed to date in other crystal structures of membrane proteins (Fig. S1B). A galactolipid was tentatively modeled into the intercalating electron density, in compliance with the notion that the uncharged heads of the galactolipids enable their penetration through the thylakoid membrane, as was demonstrated in the crystal structure of cyanobacterial photosystem II [63]. We expect that our preparation and crystallization approach would be useful for future investigations aimed at exploring the effect of lipids on the functionality of membrane proteins. Moreover, it could be highly effective for studying and determining the structure of the a-c complex of the FoF 1-ATPase.

Despite the high sequence identity between the c subunit in the green pea and the c subunits of other photosynthetic organisms (e.g., subunit-c of spinach spp. [12] and *S. platensis* [24], both displaying more than 85% identity to the green pea subunit-c), there are distinguishable structural differences among them. The
proportions of the *P. sativum* c-ring (height/width 60.5Å/60.5Å) differ slightly from those of its spinach homologue (65Å/58Å, respectively [12]), while its cyanobacterial homologue, with 15 monomers on the ring, is similar (65Å/65Å, [24]). Similarly, the coordination pattern at the proton-binding site in the pea c-ring resembles that in the cyanobacterium (Fig. 2) yet differs from that of the spinach homologue. In the latter structure, additional hydrogen bonding was detected between the hydroxyl groups of Thr64 with Oe1 of Glu6, and Gln28 was not detected in the electron density map [12]. Recent MD simulations indicate, however, that the original coordination of the proton-binding site is unstable, and therefore rapidly incorporates the bacterial-like coordinates [29]. In sum, the FoF1-ATPase c subunits derived from the chloroplasts of higher plants and from their ancestors, the oxygenic photosynthetic bacteria, share a chemical coordination and a hydrogen bond network that stabilize proton binding by the essential glutamate on subunit c.

**Network model to assess the dominant elastic modes of the c-ring**

The elastic network computational approach we used was based on the coarse-grained topology of the protein. It accounts only for the Cα-atoms and yields the eigen-modes of an undamped elastic network. In several cases it has been demonstrated that the few dominant (slow) eigen-modes observed for a given protein can be associated with large-scale and long-range functional movements that are pivotal for that protein’s mechanism [33,35]. In a real protein that is embedded in a solvent (the viscous membrane in

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**Figure 4. Motion Type I.**

A. Dynamical correlation between all residues in motion type I, derived from GNM. The correlation values range from blue to red, indicating negative and positive dynamical correlation, respectively, according to the scale. The different chains are marked on the matrix. B. The GNM dynamical correlation is mapped onto the c-ring. The c-ring is shown in cartoon representation and is viewed from the stroma. C & D. The ANM modes corresponding to motion type I. ANM1 (panel C) and ANM3 (panel D) are shown as cartoons and viewed from the lumen and stroma, respectively. The deformations are colored from gray to blue; arrows indicate the direction of motion and dotted circles mark the extreme deformations. In both modes, the c-ring expands and contracts, with oppositely correlated monomers moving towards the ring center, while the rest of the monomers move outwards. This results in an elliptic conformation.

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The mechanism of rotary proton translocation and torque generation relies on the interaction between the ring of c-subunits and subunits a and b’. The interplay between the conformational fluctuations of the c-ring and subunits a and b’ has remained to be characterized in detail. It has not yet been determined whether this interplay is mainly governed by (i) local conformational fluctuations (e.g., of residues in the ion-binding pocket of subunit c [64], or of the five helices of subunit a), or (ii) more global movements of the c-ring, as emphasized in this work. In the following, we tentatively correlate the three classes of dominant global modes of the c-ring with its rotary function in F0F1 and suggest a possible functional role.

**Motion of type I**

The contraction and expansion of the ring deform the ring elliptically (Figs. 4C and 4D, Movies S1 and S2). Expansion of the ring towards the a-subunit could favor closer apposition of the ion binding pocket on subunit-c towards the essential arginine on subunit-a. This might strengthen the electrostatic interactions between the unprotonated Glu61 and the arginine (Fig. 7A, right). On the other hand, the apposition of the flat face of the ring might facilitate the contact of Glu61 for protonation/deprotonation through either of the proton half-channels (Fig. 7A, left). The deprotonation of one Glu through one half-channel together with the protonation of the previously deprotonated Glu on the adjacent monomer on the ring implies changing the c-subunit copy that interacts electrostatically with the essential arginine on subunit a. In other words, this motion advances the rotation of the c-ring by one step.

**Motion of type II**

Motion of type II twists the stroma- and the lumen-facing surfaces against each other around the ring’s axis (Figs. 5 and 7B, Movie S3). It is conceivable that this type of deformation contributes to the previously established elastic buffer between F0 and F1 [17,19,20,21]. The latter studies do not establish the extent to which subunits γ or ε of F0 or the c-ring contributes to the high elastic compliance of this buffer. Based on the present analysis, we propose that the twisting deformation of the c-ring (Fig. 5) is part of the elastic buffer between ATP synthesis and proton transport (Fig. 7B).

**Motion of type III**

Motion of type III bends and stretches the structure as displayed in Figures 6B and 7C (see Movie S4). This motion is expected to affect the exposure of Glu61, situated at the hinge point. Upon proton release and before binding to subunit-a, negatively charged Glu61 could possibly become exposed to the membrane environment, which is thermodynamically unfavorable. The bending of the helices around the hinge position (Fig. 6B and Movie S4) could thus partially shield the charged acidic residue from the hydrophobic lipids, stabilizing this intermediate state. We suggest that Ala58, Ile65 and Tyr66 might play key roles in this process, as these are highly conserved residues around Glu61 according to our conservation analysis (Fig. 1C). Specifically, hydrophilic Tyr66 might change its original interaction with Glu61 (Fig. 2), masking it from the hydrophobic environment via its bulky ring (Fig. 7C). Interestingly, when simulating a deprotonated state of the glutamate, Pogoryelov et al. observed that the helix carrying the deprotonated glutamate is more strongly kinked [64], which is compatible with the present analysis. Our results, however, cannot account for straightening of an adjacent helix encompassing a protonated glutamate, also demonstrated by the above researchers’ simulations. The stretching of the helices, on the other hand, could increase the exposure of Glu61 towards the membrane. This, in turn, could position the deprotonated Glu61 closer to the essential positive charge on subunit-a (Figs. 6B and 7C).

**Conclusion**

Elastic network analysis of the c-ring of the FoF1-ATPase demonstrated five dominant modes of motion, and we interpreted...
the roles of these modes in the interaction of the c-ring with its counterpart in Fo (the a and bb’ subunits) and with subunits γ and ε of F1. Thus, these modes affect (i) the dynamics of sliding versus binding of c relative to abb’, (ii) proton transfer between c and a, and across the membrane, and (iii) the elastic torque transmission between Fo and F1. The coarse-grained elastic network formalism yields global modes of elastic vibrations. For a protein that is embedded in a solvent (here the membrane), these vibrations are overdamped, and the harmonic dynamics are converted into stochastic fluctuations. We suppose that the relaxation times are shorter than the transit time of proton transfer, which is approximately 10^{-4}s (at 200 mV driving force) [31]. Although the conformational fluctuations may facilitate rotary proton transfer and torque generation by Fo, they do not limit the rate, as evident from the ohmic character of proton conduction by Fo [31].

Figure 6. Motion Type III. A. The c-ring structure is colored according to the GNM-derived dynamical correlation, with positive-to-negative correlation colored according to the red-to-blue scale. Four main dynamical elements are identified, mapped to the lumen- and stroma-facing halves of the monomers at opposing sides of the ring (marked by red and blue shading). It is apparent that the stroma-facing halves are negatively correlated with their lumen-facing halves, while positively correlated with the lumen-facing halves of monomers situated at the opposing side of the ring. The left-hand side shows a side view, while the right-hand side displays stroma and lumen views. B. The deformations of the corresponding ANM motion (ANM6). The structure is colored according to the correlation of the four main dynamical elements identified in panel A. Left: ANM deformations, ranging from white to red or from white to blue, with the direction of motion marked. Right: the two extreme deformations, corresponding to the two potential directions of this type of motion, with the main dynamical elements colored red or blue according to their correlation. These deformations describe a bending and stretching motion originating from the hinge at the ring center, with bending of the helices occurring at one side of the ring, while stretching takes place at the opposing side.

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Figure 7. Cartoon illustrating the functional interpretation of the dominant modes. Schematic presentation of the c-ring (light green), subunit-a (dark gray) and the γ-c complex (pink). Protonated Glu61 of the c-ring, the conserved Arg of subunit-a and Tyr66 are shown as gray, blue and green asterisks, respectively, with unprotonated glutamates as red asterisks. A. Motion type I. Top view. Right: Upon ring expansion, the electrostatic interaction between Glu61 and the Arg is strengthened, while the probability of proton transfer is weakened. Left: Upon contraction, c-ring places a flat face toward subunit-a. This interaction could facilitate protonation/deprotonation of Glu61 through the two proton half-channels on subunit-a (dashed black arrows). It results in one step of counter-clockwise rotation (green arrows). The cartoon suggests three sites of interaction between subunits c and a, whereas it has been suggested that the Arg on subunit-a has two functions [65], resulting in two interaction sites. This alternative does not impair the above interpretation regarding effect of this subunit-c deformation. B. Motion type II. Side view with the thylakoid lumen below and the stroma side above. Helices are illustrated as gray cylinders, with the lumen-facing halves in lighter shade. As the γ-c sub-complex undergoes α-120° rotation (marked by pink arrows) and the c-ring rotates by a smaller step [21], the twisting motion of the stroma-facing helical halves (colored dark gray) is suggested to serve as an elastic buffer for smooth torque transfer between F0 and F1 (marked in directions 1 and 2 by black arrows). The opposite rotational directions illustrate the two potential functionalities, i.e. hydrolysis and synthesis. C. Motion type III. Same view as panel B, with TM2 illustrated (light gray). 1. As the structure stretches towards the left side, Glu61 of the leftmost subunit becomes more exposed to the membrane, which could enhance its interaction with the Arg of subunit-a. 2. Bending of the structure masks Glu61 from the membrane, with potential contribution of Tyr66. Again, this motion could facilitate proton exchange between the two residues.

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Supporting Information

Figure S1 Crystal contacts and packing. The c-ring is viewed in cartoon representation. A. Crystal contacts between the c-ring (blue) and its adjacent symmetry mates (green), with interactions of either the stroma loops or the N- and C- termini. B. Crystal contacts at the hydrophobic region between adjacent rings, mediated by density corresponding to single lipid molecule. A digalactolipid is modeled within the lipid density. The hydrophobic moiety of the lipid is situated exactly at the predicted hydrophobic core of the membrane (Fig. 1C). Simulated annealing omit map (Fo–Fc) contoured at 1σ.

Figure S2 Electron density map of the proton binding site. Side view of the c-ring, with the lumen below. The binding site residues are shown as sticks, with their attributed electron density shown as mesh. Density map (2Fo–Fc), contoured at 1.3σ.

Figure S3 Comparison of the slowest types of motion of the green pea c-ring, c-ring of Bacillus pseudofirmus (PDB ID 2v2v) and c-ring of the bovine F1-c8 subcomplex (PDB ID 2xnd). The deformations of the corresponding types of motion (Table S1) are shown, colored by their GNM-derived cross-correlations, according to the color bar below, with negative (blue) to positive (red) correlation ranges between -1 and 1. Arrows indicate the direction of motion. This comparison shows that although the rings are of different sizes and shapes, their three slowest types of motion correspond to each other. Similar results were obtained for the rest of the rings (Table S1). Note that for PDB ID 2xnd, the order of modes differs from that of the green pea c-ring, although the types of motion are the same (Table S1).

Figure S4 Association of GNM and ANM modes. As all types of motion consisted of the same hinge regions (Fig. 3A), we matched the GNM and ANM modes using their inter-residue cross-correlations. For motion type I, derived from the average GNM1-2 mode, ANM1 displayed a very similar cross-correlation distribution. The exact same matrix was observed for ANM2, ANM3 and ANM4 (data not shown), indicating that these modes correspond to GNM1-2 as well. The inter-residue correlation of GNM3 (motion type II) was matched to ANM5, whereas GNM4-5, representing motion type III, was associated with ANM6, as well ANM7, ANM8 and ANM9, manifesting the same motion.

Table S1 Normal mode analysis: GNM and ANM.

References


