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BIOPHYSICS

Hypotheses and Perspectives

Vol. 7, pp. 99–104 (2011)
doi: 10.2142/biophysics.7.99

Stabilization of Fo/Vo/Ao by a radial electric field

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Received July 8, 2011; accepted September 26, 2011

The membrane domain of rotary ATPases (Fo/Vo/Ao) contains a membrane-embedded rotor ring which rotates against an adjacent cation channel-forming subunit during catalysis. The mechanism that allows stabilization of the highly mobile and yet tightly connected domains during operation while not impeding rotation is unknown. Remarkably, all known ATPase rotor rings are filled by lipids. In the crystal structure of the rotor ring of a V-ATPase from *Enterococcus hirae* the ring filling lipids form a proper membrane that is lower with respect to the embedding membrane surrounding both subunits. I propose first, that a vertical shift between lumenal lipids and embedding outside membrane is a general feature of rotor rings and second that it leads to a radial potential fall-off between rotor ring and cation channel, creating attractive forces that impact rotor-stator interaction in Fo/Vo/Ao during rotation.

Key words: ATP synthase, membrane protein, electrochemical gradient, transmembrane electric field

Rotary ATPases (F-, V- and A-ATPases) are universal energy converters central to the energy metabolism of all cellular life. They either utilize an electrochemical potential in the form of proton or sodium cation gradients across a biological membrane to make ATP from ADP and P_i or pump protons or sodium cations across a biological membrane at the expense of ATP hydrolysis[1–3]. Rotary ATPases are organized in two domains: a water-soluble, cytosolic domain which is termed F_1, V_1 or A_1, and a membrane-embedded domain which is termed F_o, V_o or A_o. Energy conversion in rotary ATPases is achieved through the combination of two opposing rotary motors, which are connected by a central shaft and a variable number of lateral stalks[4]. While the soluble F_1, V_1 or A_1 domain harbors catalytic binding sites for ATP, the conversion of electrochemical energy is catalyzed by the membrane-bound F_o, V_o or A_o motor. See Figure 1 for a cartoon depicting the general organization of rotary ATPases.

Direct observation of mechanical rotation in F_1 and V_1 motors was achieved through high speed video microscopy[5,13]. Similar single molecule observation of F_1 powered rotation were made for the detergent solubilized F_oF_1 holoenzyme[6]. However, later studies showed the rotor stator interface in F_o to be rendered non-functional in the detergent solubilized F_oF_1 complex[7–9]. A further single molecule study in detergent solubilized F_oF_1, though detecting rotation in molecules sensitive to the addition of the F_o inhibitor oligomycin, did this only in very few molecules[10]. In contrast to the apparently compromised functionality of F_o in the detergent solubilized F_oF_1 complex, FRET measurements of the rotational movement of the F_o rotor ring against the F_o stator powered by both ATP hydrolysis and proton motive force did not indicate such instability[11,12]. These single molecule observations of rotation in the F_oF_1 complex impressively showed the notion of mechanical rotation as an intermediate in the conversion from electrochemical to chemical energy to be valid and at the same time indicate the importance of an intact membrane for the stable functioning of the F_oF_1 complex.

In the cytosolic domain of rotary ATPases, the three catalytic domains of the stator part are surrounding the central

Abbreviations: ATP (adenosine triphosphate), FRET (fluorescence resonance energy transfer), pdb (protein data bank), pmf (proton motive force), AFM (atomic force microscopy)

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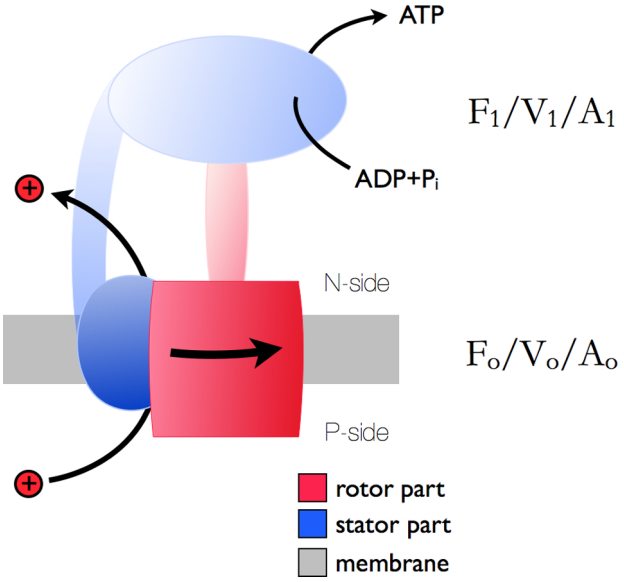

Figure 1 Schematic representation of rotary ATPases. Rotor parts are depicted in red, stator parts in blue. Cation channel forming stator and membrane embedded cation transporting rotor ring are highlighted by darker hues.

rotor shaft[15–17]. This spatial arrangement ensures stability of the rotor-stator interaction during energy conversion at high processivity. In contrast to this bearing-like stabilization mechanism, the stator part is in contact with the rotor part at only one peripheral site in the membrane embedded F_o, V_o or A_o motor[18–20]. In an early proposal of this arrangement in F_o it was noted that the stable assembly of rotor and stator during rotation requires explanation[21]. Generally, the membrane bound electrochemically driven rotary stepping motor consists of a cation binding rotor ring and a peripheral cation channel forming subunit, termed F_o-a, V_o-a, A_o-a or adjacent subunit. The rotor ring is formed by a ring-like arrangement of multiple pairs of transmembrane alpha helices. One transmembrane alpha helix of each pair faces the inside and one the outside of the rotor ring. Rotor rings contain a species-dependent number of cation binding sites that face the hydrophobic core of the surrounding membrane. The best studied rotor rings are those of F-ATP synthases. In F-ATP synthases rotor rings consists of a multiple of c-subunits, hence also named c-rings. Each c-subunit is formed by one hairpin alpha helix pair and contains one cation binding site.

The negative potential side of a rotary ATPase embedding membrane, often identical to the cytoplasmic side, is named N-side. The positive potential side, in bacteria and archae identical to the periplasmic side, is named P-side. Access to the cation binding sites from both sides of the membrane is thought to be given by water-filled half-channels at the interface of the rotor ring and the cation channel forming adjacent subunit[4,21]. Biochemical studies employing silver cation accessibility assays provide evidence for the existence of water access half-channels at the rotor-stator interface[22].

During catalysis, the rotor ring rotates against the cation channel forming subunit at a rate of up to 700 revolutions per second[10,11]. That even faster rates of rotation are possible was shown in a careful study on F_o in chromatophores. Despite the absence of both peripheral and central stalk, F_o remained tight against proton leakage, showed a linear dependence between rotation rate and transmembrane potential and remarkably no signs of saturation with increasing driving force[23]. This clearly demonstrates the functionality of the rotor-proton channel interface in F_o not to depend on the presence of F_1, but rather on itself and its embedding membrane.

A recent cryo-electron microscopy study on a detergent solubilized bacterial V-ATPase from *Thermus thermophilus* suggests the contact surface between Vo-a and rotor ring to be very small[24]. If the small contact surface is a physiological relevant feature or a consequence of the missing membrane remains to be shown. Detailed structural information on the molecular architecture of the cation channel forming subunit and especially its interface with the rotor ring is not available. Thus, the nature of the interaction that allows constant rotation of cation channel and rotor ring against each other while providing stability and tightness remains an intriguing problem[14].

Hypothesis

The hypothesis presented here is that, first, a vertical shift of lipids filling rotor rings towards the P-side is a general feature of rotary ATPases. And, second, that the resulting proximity of the P-side half-channel to the water filled inside of rotor rings leads to a radial potential fall-off that impacts the interaction of the rotor-stator interface.

Rotor rings of rotary ATPases contain a shifted inner membrane

A wealth of structural and biochemical data is available for rotor rings from various, evolutionary distant species. Atomic models from crystal structures have been determined for the c_8-ring of the bovine mitochondrial F-ATP synthase, the c_{10}-ring of the mitochondrial yeast F-ATP synthase, the c_{11}-ring of the sodium powered F-ATP synthase from *Ilyobacter tartaricus*, the c_{13}-ring of a thermoalkaliphilic F-ATP synthase from the *Bacillus* species, the c_{15}-ring of the F-ATP synthase of the photosynthetic cyanobacteria *Spirullina platensis* and the homologous K_{10}-ring of the sodium pumping V-ATPase from the bacterium *Enterecoccus hirae*[25–31].

Early biochemical and AFM studies on the c_{11}-ring from *Ilyobacter tartaricus* demonstrated that it is filled with a phospholipids containing plug protruding from the P-side of the ring[32]. Similarly, a later AFM study on two-dimensional arrays of membrane reconstituted *Bacillus* c_{13} rings revealed a lipid plug protruding from the P-side[33]. Both studies were

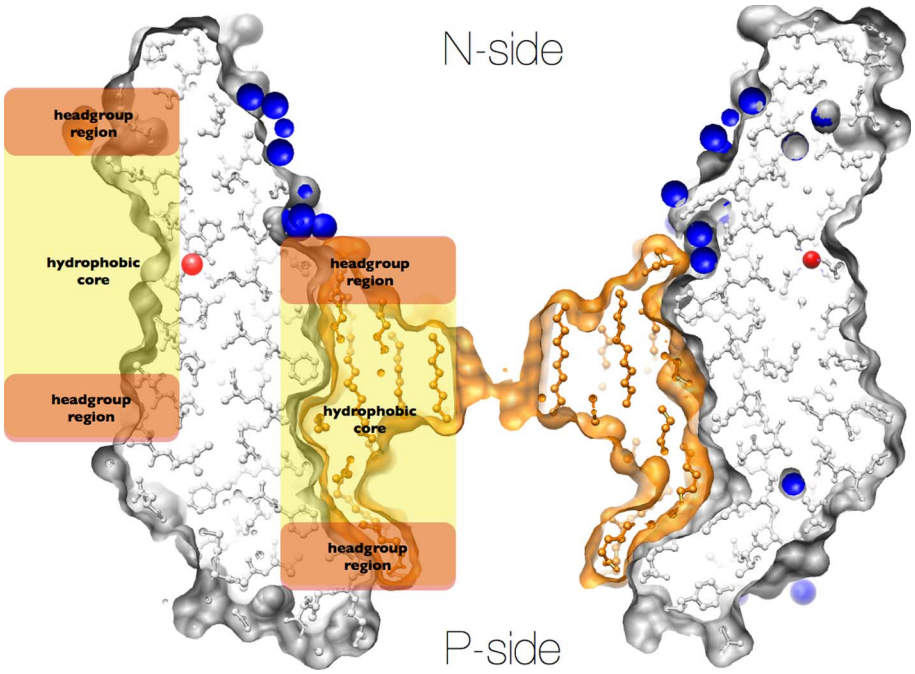

Figure 2 Central slice of the K-ring from *Enterecoccus hirae* (pdb 2BL2) perpendicular to the membrane plane. Protein is depicted in white ball&stick, protein surface in light grey, sodium as a red sphere, water as blue spheres and lipid as ball&stick and surface in gold-orange. Boundaries of the inside membrane and the expected position of the outside membrane are indicated by overlayed boxes. Notice the coincidence of height between outside bound sodium and inside lipid headgroups.

conducted on rotor rings reconstituted into artificial membranes, leaving room for speculation concerning the situation in the intact enzyme in its native membrane. However, a photo-cross-linking based study on intact *E. coli* F-ATP synthase in its native membrane demonstrated that the inside lumen of the rotor ring is filled with phospholipids[34]. These observations make it plausible that in general the rotor ring′s inner lumen is sealed by lipids in its native environment and that the relative position of these lipids is biased towards the P-side.

In the high-resolution crystal structures of the same c_{11}-ring from *Ilyobacter tartaricus* and the c_{15}-ring from the cyanobacterium *Spirullina platensis*, lipids are absent from the inner lumen of the rotor ring. A molecular dynamic simulation of both rings that included outer and inner membrane, however, suggests a shift of the inner membrane with respect to the surrounding membrane towards the P-side[35]. So far, the only structural insight on the inner membrane of a rotor ring at atomic resolution was given by the serendipitous co-crystallization of the K-ring from the bacterial V-ATPase of *Enterococcus hirae* with native lipids bound to the rotor ring′s lumen[31]. This structure unequivocally shows the ring to be filled with lipids that form a proper lipid bilayer with upper and lower leaflet . Very much like the lipid plug of the c_{11} and c_{13} rings, the K-ring bilayer is located at the periplasmic end of the ring inside which results in a shift of the inner lipid membrane towards the P-side (Fig. 2). In this arrangement, the lipid head groups facing the N-side of the inner membrane align exactly to the height of the sodium cations bound at the outside of the ring. Thus, the cytoplasmic side of the rotor ring lumen is filled with water. Crystal structures of F_1-c-ring complexes with the central shaft bound to the rotor ring show that the upper lumen of the rotor ring is not occupied by protein and is accessible from the cytoplasm[25,26,36]. Therefore, the upper inner lumen of these rotor rings is electrochemically connected to the cytoplasmic bulk solution.

A cross-section of the K-ring from *Enterococcus hirae* shows the outside-bound sodium cation to be horizontally in line with alternating charges of a negatively charged lipid head group phosphate, the terminal positive nitrogen of lysine 32 and the partially negative charged water oxygen (Fig. 3A, B). This arrangement could strongly influence the local dielectric environment. The distance between sodium cation and the closest structural water on the inside of the rotor ring is less than 12 Ångstrom (Fig. 3B). This is much shorter than the phosphate-to-phosphate distance of more than 33 Ångstrom observed between lipid molecules in the two leaflets of membranes surrounding bacteriorhodopsin (pdb 2AT9) or aquaporin0 (pdb 2B6O)[37] and the inner membrane of the K-ring (pdb 2BL2). It is also considerably shorter than the distance between neighboring sodium ions in the K-ring of 20 Ångstrom or the effective distance of ~18 Ångstrom between proton acceptor/donor sites proposed for Fo-a[23].

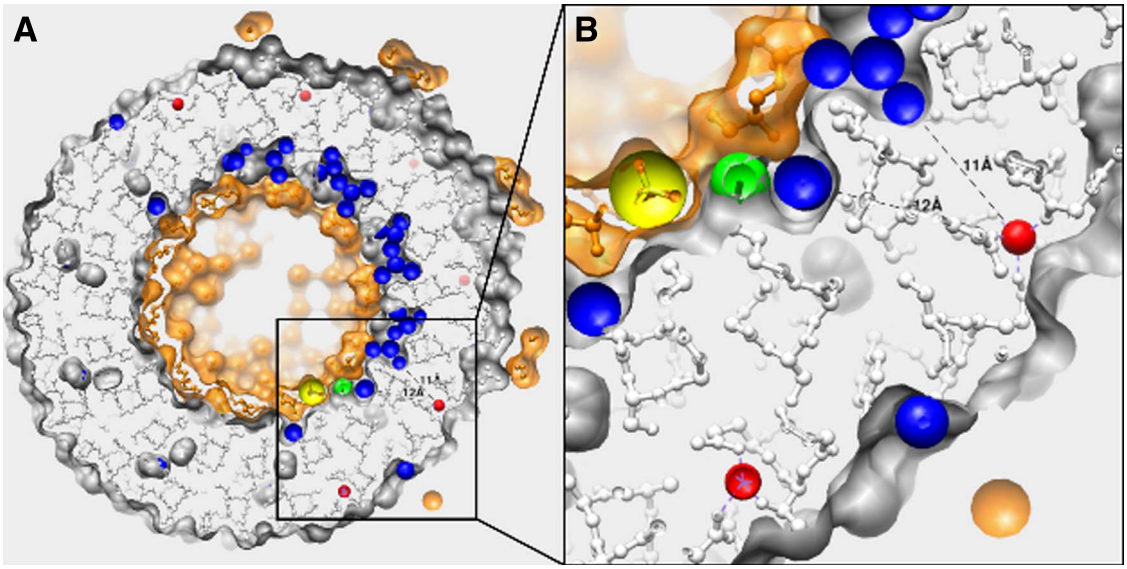

Figure 3 (A) Cross-section through the K-ring horizontal to the membrane plane at the height of the sodium binding sites. (B) Close-up of the same cross-section. The proximity of the bound sodium to structural water of the ring inside is indicated by broken lines and the horizontal line-up of charges by the colored spheres of a lipid head group phosphate (yellow), the terminal nitrogen of lysine 32 (green) and a structural water (blue).

A possible radial potential fall-off might stabilize rotor-stator interaction in the membrane

A water-filled half-channel in the stator of the Fo/Vo/Ao motor provides access to rotor ring cation binding sites from the P-side[22,38,39]. If the membrane shift observed in the K-ring is indeed a structural feature of rotor rings in general, then the P-side half-channel lies in proximity to the water-filled cytoplasmic inside of the rotor ring. Generally, location and direction of a potential fall-off across a membrane are determined by the geometry of the membrane and the membrane′s local dielectric constant. Thus, the membrane potential is expected to fall off where the distance across a region of low dielectric constant is the shortest, i.e. the insulation is thinnest. Along similar arguments of distance and geometry, a horizontal membrane potential fall-off between two half-channels has been incorporated as an important element for torque generation in a numerical model of the F_o motor[40]. The apparent proximity between P-side half-channel and cytoplasmic inside of the rotor ring makes it likely that at least a partial membrane potential fall-off is occurring radially between them (Fig. 4). I hypothesize that a potential fall-off between adjacent subunit and rotor ring inside will be accompanied by an electric field. Such an electric field will exert force on charges at the interface between adjacent subunit and rotor ring, possibly providing attraction between rotor and stator. This mutual attraction could compensate for frictional forces during rotation and thus stabilize the complex. Importantly, increased rotation due to a higher membrane potential would be accompanied by stronger attraction. Thus the stabilization of the stator-rotor interface is conceived to be achieved by two complementary forces: a “resting” interaction, e.g. by van der Waals forces, and a secondary one induced by a radial potential fall-off between P-side half-channel and rotor ring inside.

In this context it is noteworthy that the voltage-sensing domain of voltage activated ion-channels has been shown to manipulate the membrane potential fall-off by a combination of change in local dielectric constant and geometry to focus the electric field on hydrated arginine residues that exert the necessary force for the opening of the ion pore[41].

Candidates for charged residues experiencing force through an electric field between P-side half-channel and rotor ring are the essential arginine of the adjacent subunit and the cation binding glutamate or aspartate of the rotor ring. With one elementary charge at a transmembrane potential of 200 mV, one charged residue would have an electric potential energy of 0.2 eV which is equivalent to the energy of a hydrogen bond of medium strength at 20 kJ/mol. Therefore, over the time of one full rotation, the frictional forces between stator and a c_{10} ring would be counteracted by an equivalent of more than 10 hydrogen bonds from the charged residues. Even if assuming that only a fourth of the membrane potential falls off radially, the forces generated are still in the range of protein-protein interaction. Moreover, forces generated from a radial electric field would stem not only from pre-existing charged residues, but also from non-permanent charges induced by the electric field itself. Apart from creating attractive forces which are supporting the stability of the rotor-stator complex during rotation, the putative radial electric field may facilitate the rotamer conformational changes in cation binding glutamate or aspartate that are proposed to be essential for uptake and release of cations[42]. This facilitation could effectively enhance the

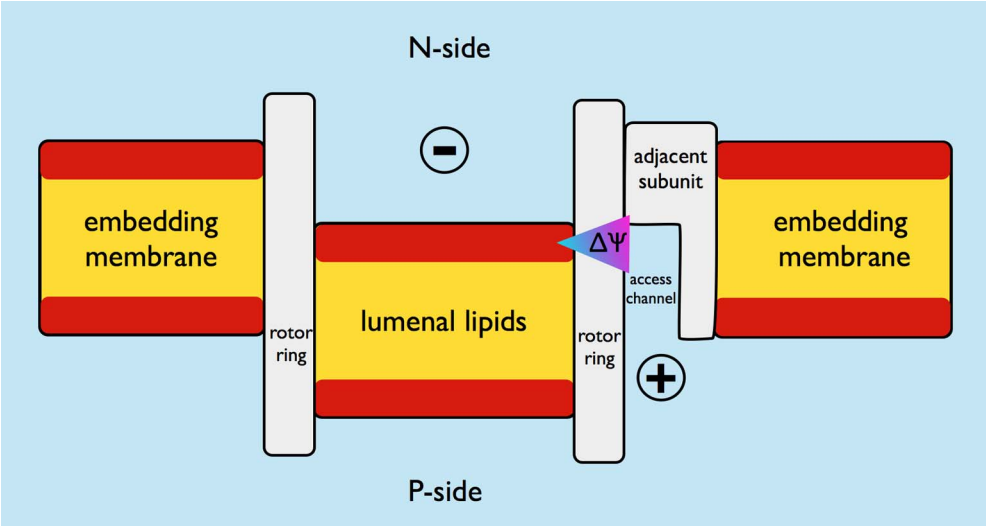

Figure 4 Cartoon illustrating how horizontal proximity of the cation binding site and bulk solution on the inside of the rotor ring could result in a radial membrane potential fall-off, indicated by a purple triangle, at the interface of the rotor ring and the adjacent subunit. Regions of high dielectric constant are depicted in blue for bulk solution and red for lipid headgroups. Regions of low dielectric constant are shown in grey for protein and in yellow for the hydrophobic membrane core.

P-side half-channel's role as a Mitchell′ proton well[43]. Additionally, the closeness of lipid head groups on the inside of the rotor ring to the site of cation uptake/release possibly also lowers the desolvation barrier at the a/c interface discussed in[44].

A straightforward way to falsify the importance of ΔΨ on the stabilization of rotor-stator interaction in Fo/Vo/Ao would be to perform ATP synthesis with a pmf in which the ΔΨ component is in reverse. In other words both lower pH and negative potential on the P-side. Another possible experiment could be the introduction of residues in the rotor ring of the Fo domain that either shorten or lengthen the radial distance between cation binding site and rotor inside. A thickening is expected to have a detrimental effect on the stability of Fo. A further interesting experiment would be to map sites of potential fall off on the rotor ring through the use of electrochromic fluorophores such as Di-1-ANEPIA[45]. The hypothesis predicts the detection of changes in the local electric field that are more pronounced on the inside of the rotor ring at the height of the cation binding site than at loop and termini regions. Furthermore, single molecule experiments on the Fo complex that pull rotor ring and stator apart could be used to probe if a membrane potential has or has not a stabilizing effect. Computational methods could be used to calculate the energetic consequences a radial horizontal field could have on the open rotamer conformation of the cation binding glutamate or aspartate at the rotor-stator interface.

Eventually, it will be necessary to elucidate the molecular architecture of the rotor-stator interface including the inner and the surrounding membranes. The determination of the exact position of a lipid bilayer in which a membrane protein complex is embedded may be ambiguous from the structure of the protein alone. Thus, direct structural insights from type I three-dimensional crystals or two-dimensional crystals that include membranes together with the protein structures will be essential for a complete understanding of the electric motor of rotary ATPases.

Acknowledgements

I want to thank Michael Börsch, Hiroyuki Noji, Kaoru Mitsuoka, Wolfgang Junge, Daniela Stock and Boris Feniouk for their stimulating discussion and encouragement. I also want to express my sincere thanks to Florian Hauer and Kathy Gelato for discussion and critical reading of the manuscript. This work was supported by the Special Coordination Fund for Promoting Science and Technology of MEXT, Japan.

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