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TOPICAL REVIEW

Opposite Rotation Directions in the Synthesis and Hydrolysis of ATP by the ATP Synthase: Hints from *a* Subunit Asymmetry

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Abstract The ATP synthase can be imagined as a reversible H^+-translocating channel embedded in the membrane, F_O portion, coupled to a protruding catalytic portion, F_1. Under physiological conditions the F_1F_O complex synthesizes ATP by exploiting the transmembrane electrochemical gradient of protons and their downhill movement. Alternatively, under other patho-physiological conditions it exploits ATP hydrolysis to energize the membrane by uphill pumping protons. The reversibility of the mechanism is guaranteed by the structural coupling between the hydrophilic F_1 and the hydrophobic F_O. Which of the two opposite processes wins in the energy-transducing membrane complex depends on the thermodynamic balance between the protonmotive force (Δp) and the phosphorylation potential of ATP (ΔG_P). Accordingly, while Δp prevalence drives ATP synthesis by translocating protons from the membrane P-side to the N-side and generating anticlockwise torque rotation (viewed from the matrix), ΔG_P drives ATP hydrolysis by chemomechanical coupling of F_O to F_1 with clockwise torque. The direction of rotation is the same in all the ATP synthases, due to the conserved steric arrangement of the chiral *a* subunit of F_O. The ability of this coupled bi-functional complex to produce opposite rotations in ATP synthesis and hydrolysis is explained on the basis of the *a* subunit asymmetry.

Keywords *a* subunit asymmetry · F_1F_O-ATPase · Torque generation · Protonmotive force

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Introduction

The F_1F_O-ATPase, or ATP synthase (EC 3.6.1.3), is an enzyme complex present in the inner mitochondrial membrane, in the plasma membrane of bacteria and in the thylakoid membranes of chloroplasts. The F_1F_O complex synthesizes ATP from ADP and P_i by dissipating the protonmotive force (pmf or Δp) or in some bacteria the sodiummotive force (smf). However, under peculiar physiological conditions, it can also hydrolyse ATP and exploit the free energy contained in the phosphoanhydride bond of the adenylic nucleotide to act as a proton pump and to generate a transmembrane ionic gradient (Yoshida et al. 2001; Capaldi and Aggeler 2002; von Ballmoos et al. 2009; Walker 2013). The hydrolytic function is usually associated with lack of oxygen (Grover and Malm 2009). For instance, some fermenting bacteria hydrolyze ATP to make a proton gradient, which they exploit for nutrient transport and other cell requirements (Deckers-Hebenstreit and Alterdorf 1996).

Studies carried out in recent times have brought to light that the F_1F_O-complex not only has the bioenergetic function to produce ATP, the energy currency of life, but it can also play morpho-functional roles in mitochondria. The supramolecular organization of F_1F_O-ATPase in dimers, as well as in long row of oligomers, forces the membrane to assume a positive curvature, thus guaranteeing the morphology of mitochondrial *cristae* (Strauss et al. 2008). The implication in mitochondrial permeability transition (Giorgio et al. 2013; Bonora et al. 2014) and the candidacy as enzyme target for antimicrobial and anticancer drugs (Johnson and Ogbi 2011; Sakthivel 2012; Nesci et al. 2014) *de facto* make the F_1F_O-ATPase a sort of boundary line between cellular life and death.

Both the protonmotive-driven synthesis of ATP and the H^+ pumping activity driven by ATP hydrolysis are made

possible by the two coupled rotary motors of F_1F_O-ATPase: one chemical known as F_1 (factor 1) and one electrical known as F_O (factor oligomycin) (Junge et al. 2009; Dabbeni-Sala et al. 2012). These two main structural sectors of the F-ATPase have distinct tasks and structures; the hydrophilic domain F_1 binds adenine nucleotides and inorganic phosphate (Pi), whereas the membrane-embedded hydrophobic F_O domain constitutes the ion-translocating portion. The two domains are connected by a central stalk and a peripheral stalk (Devenish et al. 2008). The former is the key rotary element which transfers energy from F_O to F_1 and vice versa (Gibbons et al. 2000). The latter stalk, which extends laterally for the entire enzyme complex height, acts as a stator to counteract the tendency of the catalytic sites to rotate driven by the central stalk (Walker and Dickson 2006). The Δp energy is exploited by the F_1F_O-ATPase to translocate ions through F_O by a mechanism of protonation–deprotonation of acidic aminoacid residues and electrostatic attractions at the *a*/*c*-ring interface (Pogoryelov et al. 2010). This mechanism allows an angular progression of *c*-ring which creates a torque generation, in turn making the central stalk rotate. In this way, the energy of the transmembrane electrochemical gradient is mechanically transmitted to the hexamer of α-β subunits of F_1 involved in the catalytic cycle of ATP (Boyer 1993). The catalytic cycle is reversible, namely it results in either ATP synthesis or ATP hydrolysis, due to this unique energy transmission F_1F_O-ATPase mechanism leading to synthesize or hydrolyze ATP according to the functional state of mitochondria. The catalysis is coupled to proton translocation by torque generation associated to two opposite directions of rotation of the central stalk: counterclockwise (when viewed from the mitochondrial matrix side toward the cytosol) in the ATP synthesis and clockwise in ATP hydrolysis. This immutable mechanism, the same for all F_1F_O complexes, whose fixed directionality seems guaranteed by the chiral arrangement of two semi-channels of *a* subunit, plays a specific functional role in the wonderful structural arrangement of this splendid molecular machine (Boyer 1997). Indeed, chirality is a quite common feature of biological structures (Gujarro and Yus 2009), due to the complexity and the abundance of stereocenters in biomolecules and to the stereoselectivity of enzymes involved in their building and manipulation. Additionally, any directional motion places functional limits on the symmetry of protein machinery, in turn mainly favored in oligomers (Goodsell and Olson 2000). Apparently, life evolved toward chirality. Not often, however, the link between a defined configuration and its functionality in biological mechanisms is immediately clear, leading to intriguing speculations.

In F-ATPases, the asymmetry of F_1 hexamer (Sun et al. 2004) as well the structural differences between the adjacent α/β and β/α interfaces and the interaction with γ subunit, which was first involved in the rotation directionality (Uchihashi et al. 2011;Watanabe and Noji 2013) have been already considered in the rotary mechanism of the catalytic function. Being the intimate mechanism of the coupling between rotation and catalysis in the ATP synthase still partially obscure, in spite of the wealth of studies (Boyer 1988; Yoshida et al. 2001; Capaldi and Aggeler 2002, Dimroth et al. 2006; Devenish et al. 2008; Junge et al. 2009; Mitome et al. 2010; Uchihashi et al. 2011; Dabbeni-Sala et al. 2012; Jonckheere et al. 2012; Sielaff and Borsch 2013; Walker 2013; Watanabe and Noji 2013), the handedness of the subunits involved can lead to a better understanding of the relationship between structural arrangement of biomolecules and their biological role. In this context, the model of *a* subunit functioning here described may represent a contribution to achieve a satisfactory and reasonable enlightenment of the mysterious link between rotation and catalysis in the enzyme complex which plays a key role in cell life and death.

Role of the *a* Subunit in Establishing the Direction of Rotation

In a few words, F_O can be defined as a rotary electrochemical generator driven by proton flow. The extraordinary ability of this proton-driven engine to convert transmembrane Δp in the universal chemical energy source lies in the ingenious system for generating torque to ion passage through F_O, and transmitting such rotation to F_1 (Dimroth et al. 2006). The core of F_O consists of an oligomeric ring of hydrophobic *c* subunits, namely multiple copies of two membrane-spanning hairpin-shaped α-helices (Stock et al. 1999). The *a* subunit, whose structure has not been completely elucidated yet (Vik and Ishmukhametov 2005; Walker 2013), connects the so-called *c*-ring to the peripheral stalk (Baker et al. 2012). Indeed, little is known about the detailed structure of this elusive protein (Walker 2013). Charged residues in the *a* and *c* subunits seem to be essential in the coupling of ion translocation to rotation. Among these charged residues, most likely the positive charge of a guanidinium group of the highly conserved Arg which corresponds to the stator charge of *a* subunit (Elston et al. 1998) and the proton-binding sites of the carboxylic groups of Asp or Glu (depending on the species) of *c* subunits constitute suitable candidates for the whole process. The non-linear arrangement of the two aqueous semi-channels, or pathways, within *a* subunit (Angevine and Fillingame 2003) builds the molecular architecture for proton translocation. Even if the proton channeling has not been structurally defined yet, there is general consensus to imagine the

proton pathway within two non-linear hydrophilic semi tunnels built by the aminoacid side chains of the protein (Walker 2013). The protonation sites of the *c*-ring look out at semi-ion channels and they are accessible from both the negative (N) and positive (P) sides of the membrane (Junge et al. 1997; Elston et al. 1998). The Arg of the *a* subunit acts as an electrostatic barrier which splits proton access in two ways (Mitome et al. 2010). In the rotary dynamics of F_O, protons can be exchanged at the interaction surface where the rotor meets the stator. When Δp is enough high to allow ATP synthesis, the semi-channel exposed on the P-side of the membrane constitutes the way of access of protons to the carboxyl groups of *c*-ring. After the binding-site protonation, the *c* subunit becomes less polar and thus able to enter the surrounding lipid layer, maintaining the acidic residue oriented toward the center of the rotor ring in the so-called proton-locked conformation, which is energetically favored (Pogoryelov et al. 2009). At the same time, a *c* subunit with neutralized carboxyl group is exposed on the semi-channel that looks toward the N-side of the membrane. The more hydrophilic environment surrounding the proton-binding sites with respect to the lipid interface makes the carboxyl residue reorient in the open conformation pointing toward the *a* subunit (Symersky et al. 2012). In this state, the basic pH at the N-side of the membrane and the positive charge of the Arg proton barrier imply structural rearrangements which lower the pK_a of the carboxylic residue allowing proton detachment. Accordingly, the deprotonated carboxylate ($-COO^-$ form) is stabilized in the open conformation through salt bridges with the essential stator Arg, whose pK_a is high enough to maintain the protonated form of guanidinium group with charge +1. When a new proton faces the positive semi-channel at the P-side, the binding site switches again to the protonated (–COOH) form, re-oriented in the closed conformation to start a new rotational cycle (Pogoryelov et al. 2010). The interplay between the electrostatic barrier of *a* subunit, built by the positive charge of Arg, and the *c*-ring proton-binding sites represents the most attractive mechanism of F_O. This sophisticated electric co-operation "channels" the driving force of transmembrane proton flow by a simple ion path within *a* subunit that prevents proton leakage and ensures a frictionless rotation of the rotor (von Ballmoos et al. 2009).

The *a* subunit, other than representing a key component for proton transport and torque generation mechanisms, with its oscillating arrangement of the two proton-conducting semi-channels from the P-side to the middle of the lipid bilayer and again from the middle of lipid bilayer to the N-side, makes the direction of rotation of the *c*-ring mandatory. The counterclockwise rotation of F_O (viewed from the mitochondrial matrix) driven by Δp implies that the protons must be channeled downhill in only one direction, namely from the P-side to the N-side of the membrane. If the *a* subunit is imagined as localized between the observer and the *c*-ring rotor, necessarily proton must enter from the right side of the *a* subunit and exit on the left side, because structurally proton entry is on the right side and proton exit on the left side. Accordingly, the two semi-channel structures show asymmetric non-linear arrangements in which the right semi-channel turns toward the P-side of the membrane, while the left semi-channel faces the N-side (Fig. 1). The two semi-channels are featured by different electric charges and pHs so as to be extremely apt to play the role of "proton tunnel". Accordingly, the stereo-specific arrangement of the two semi-channels at the *a*/*c* interface drives proton flux in a unique and obligatory direction and consequently makes the *c*-ring rotate in only one possible direction, by ensuring at the same time the opposite rotation when proton current is reversed.

On considering the hypothesized proton translocation mechanism via coordination of hydronium ion (H_3O^+) to H^+ binding sites of *c*-ring (Boyer 1988), studies from our laboratory strongly suggest that the rate-limiting step is the H^+ delivery by H_3O^+at the F_O-*a*/*c* interface to protonate the *c*-ring carboxylate $-COO^-$ (Nesci et al. 2013). Accordingly, H^+ transport would occur by Grotthuss mechanism, namely by short H^+ hops from H_3O^+ to the adjacent water molecule. Consequently, the chain of water molecules within each semi-channel of *a* subunit must be constantly polarized to allow continuous rupture and re-formation of the covalent bond between H^+ and oxygen of water.

In summary, based on present knowledge, the lack of a plane of symmetry in the semi-channel architecture within

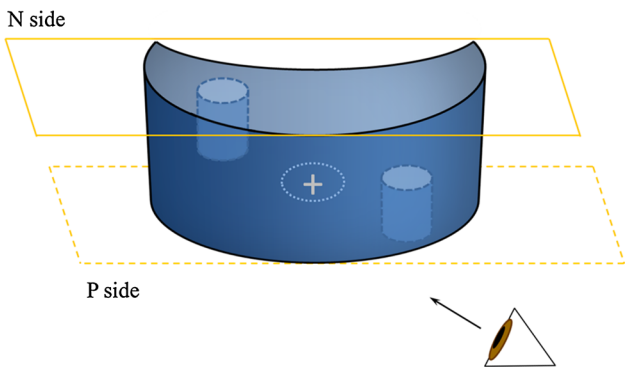

Fig. 1 Model of the asymmetric structure of the *a* subunit in which the proton pathways are illustrated as two cylindrical semi-channels. The right semi-channel (seen by the observer), looks at the positive side of the membrane and the left semi-channel looks at the negative side of the membrane. The symbol (*plus*) indicates the positive charge of arginine that acts as electrostatic barrier to protons and leads to unidirectional proton flux

a subunit is fully consistent with the obliged direction of rotation for ATP synthesis opposite to that leading to ATP hydrolysis.

Counterclockwise and Clockwise Motion for the F_1F_O-ATPase Does Not Simply Mean Synthesis and Hydrolysis of ATP

The directionality of rotation is not only essential for the two opposite catalytic functions of the F_1F_O complex but also for emerging "new" functions which confirm the centrality of the enzyme complex in cell life and death. Apparently the whole rotary mechanism has evolved toward the development of multiple controlling factors which involve several enzyme subunits and most likely complement each other.

The bioenergetic cost of cellular ATP is indirectly influenced by the relative contribution of the two components of transmembrane Δp, namely the electrical component (membrane potential, $\Delta\varphi$) and the chemical component (difference between proton concentration, ΔpH) (Watt et al. 2010). A predominant $\Delta\varphi$ in the membrane is more efficiently used by a small *c*-rings with the same principle of operation of a gear on the back wheel of bicycle (Nicholls and Ferguson 2013). Accordingly, with a small gear is necessary to give a lot of force, but only a few rides are required to make the wheel rotate. Thus, the rotation of the rotor is powered by a high $\Delta\varphi$, while the low ΔpH value supports the poor number of protons translocated across the membrane. Conversely, large *c*-rings require high ΔpH, which implies sufficient proton current to compensate for proton dissipation during the rotation of the central stalk; in this case protons are not driven by $\Delta\varphi$ (von Ballmoos et al. 2008). The number of translocated protons (which mirrors the *c*-ring stoichiometry) divided by the yield of three ATP molecules synthesized in a full catalytic cycle gives the H^+-to-ATP ratio (H^+/ATP or *i*), which defines the protons required to build one ATP molecule. A low *i* ratio is assigned to small *c*-rings, while a high *i* ratio is typical of large *c*-rings (Ferguson 2010; Silverstein 2014). At fixed ΔG_P, F_1F_O complexes featured by a low *i* value have high reversal potential threshold (E_{rev}), since $E_{rev} = \Delta G_P/i$. In this case the F_1F_O-ATPase will be more sensitive to the membrane depolarization which triggers the transition from ATP synthesis to ATP hydrolysis, and vice versa, if *i* is high, the enzyme complex will be less sensitive to the change of direction since E_{rev} is low. In other words, high sensitivity means that slight modifications of Δp can reach the high E_{rev}, whereas low sensitivity imply that a drastic drop in Δp is required to attain the low E_{rev}. These two complementary and reversible processes result from a thermodynamic equilibrium. ATP hydrolysis by the F_1F_O-ATPase is not a physiological process, but it may occur under patho-physiological conditions whenever Δp drops. A Δp drop frequently results from hypoxic conditions, as in myocardial ischemia (Grover and Malm 2009). By exploiting ATP hydrolysis, the mitochondrial F_1F_O complex functions as a proton pump to restore the transmembrane electrochemical gradient of protons and this process is regulated by a small basic protein, known as inhibitory factor 1 (IF_1). Its homo-dimeric structure has two antiparallel α-helices with the inhibitor sector in the N-terminal regions, while the C-terminal region holds together each monomer by a coiled coil (Cabezón et al. 2001). The two terminal sectors of IF_1 can simultaneously inhibit two distinct F_1F_O complexes by inserting into the C-terminal region of a β_E-subunit (empty). The fully inhibited status is attained after two consecutive 120° steps of the central stalk (γ-subunit) which changes the conformation of the β subunit from the β_E-form to the β_{TP}-form, which binds ATP, and finally to β_{DP}-form, which binds ADP. In this conformation the globular catalytic domain of F_1 is blocked by IF_1 and the F_1F_O-ATPase cannot hydrolyze ATP (Gledhill et al. 2007). The inhibition mechanism by IF_1 is reversible and unidirectional since it does not inhibit ATP synthesis. Indeed, the building of Δp reverses the rotation of the central stalk by channeling the proton flow within *a* subunit. The counterclockwise direction (as viewed from the matrix) required for ATP synthesis reactivates the enzyme causing the expulsion of IF_1 from the β_{DP}–α_{DP} interface by ratchet-like action. Therefore, the direction of rotation of the rotor determined by *a* subunit is fundamental to allow IF_1 to lock/unlock the catalytic hexamer ($\alpha_3\beta_3$) and to modulate by such intermittent mechanism the inhibition of ATP hydrolysis.

Recent advances confirm that, independently of F_O, programmed structural basis of unidirectionality lie in the $\alpha_3\beta_3$ stator ring which constitutes F_1. The conformational states of subunits are propagated unidirectionally and cyclically through cooperative interactions. The intrinsic interplay among β subunits, main constituents of the catalytic sites, strengthens the catalytic control by γ (Uchihashi et al. 2011), in turn driven by the *c*-ring. Thus, it seems likely the occurrence of distinct mechanisms which cooperate in determining the direction of rotation may represents a sort of "safety mechanism" ensuring unidirectionality.

The mitochondrial ATP synthase is able to form dimers that in turn can assemble into oligomers (Baker et al. 2012). The supramolecular organization of F_1F_O-ATPase ensures the mitochondrial morphogenesis, a key process of cell physiology (Habersetzer et al. 2013), but also optimizes its own performance. Accordingly, the dimer ribbons ensure a strong local curvature on the inner mitochondrial membrane and act as proton traps since regions featured by high membrane curvature show a significant increase in

proton density (Strauss et al. 2008). The supramolecular assembly of the F_1F_O-ATPase affects metabolism, subcellular structure, diseases and aging (Seelert and Dencher 2001). F_1F_O-ATPase dimers, held together by the respective *e* and *g* subunits, represent two mirror images with an age-dependent angle of inclination between the axes (Daum et al. 2013).

As depicted by a fascinating model, the stator-to-stator arrangement of the F_1F_O-ATPase dimers represents a putative molecular strategy to prevent counter-rotation of the peripheral stalk. According to the law of conservation of angular momentum, the peripheral stalk anchoring the catalytic subunits $\alpha_3\beta_3$ moves to the opposite direction with respect to the rotation of the rotor associated with ATP synthesis or hydrolysis. To counteract the undesired yaw generated by the rotor, the specular arrangement of the enzyme complex in dimers would result into a reciprocal torque compensation similarly to a tail rotor. Indeed, the moment of force on the F_1F_O-ATPase stator during the rotation of rotor opposes to the torsion undergone by the other counterpart stator of the dimer (Fig. 2). This equal balance of turning force between the two dimers is guaranteed by two fundamental conditions: the rotation rate of the rotor, directly proportional to the radius of the *c*-ring, which has the same size for a stated species (von Ballmoos et al. 2008; Silverstein 2014), and the direction of rotation, ensured by the asymmetric arrangement of the two semi-channels of *a* subunit. As far as we are aware, the F_1F_O-ATPase of all living organisms possesses the same semi-channel structure of the *a* subunit and consequently maintains the same rotation directions during ATP synthesis (counterclockwise) or hydrolysis (clockwise)

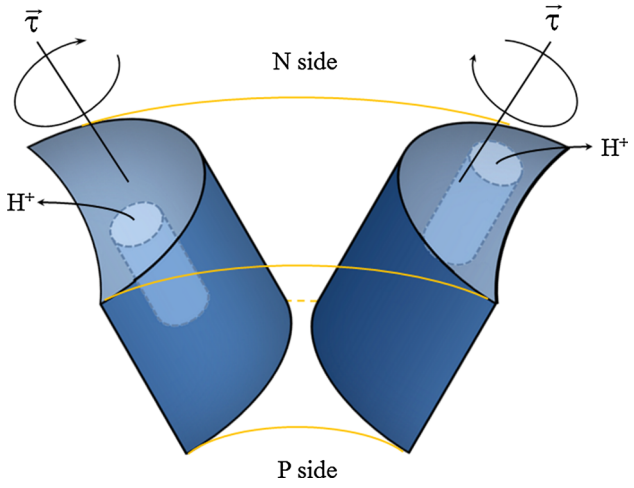

Fig. 2 Putative coupling of two adjacent *a* subunits in the ATP synthase dimer. The two moments of force (τ), generated by rotor rotations to the stators of each monomer in the supercomplex, have the same magnitude and opposite directions due to the specular structural arrangement of the two monomers

(Jonckheere et al. 2012). It seems reasonable to hypothesize that the two rotors of specularly arranged monomers in the supramolecular structure of the dimer which rotate according two opposite directions, will counterbalance the moment of force of their two stators and stabilize the supercomplex.

Conclusion

Despite the extensive studies on the F_1F_O-ATPase, the detailed functions of this complex enzyme machinery are not yet fully understood. The bi-functional rotary mechanism makes this proton engine an extraordinarily perfect molecular machine, amazingly far from any known turbine designed by the human mind. The most striking mechanism in this complex enzyme is probably that by which two simple proton-wires are able to organize and control the torque generation inside the protein. Once again chirality seems to constitute the molecular basis of a physicochemical function. Indeed, directionality is driven by multiple factors within the F_1F_O complex, but, among them, the role of the *a* subunit in ensuring the absolute configuration around its chiral arrangement is increasingly shown to be essential. Reasoning by absurd, if the arrangement of the semi-channels in F_O were reversed, all the catalytic events of the enzyme complex would occur otherwise. As stated by Einstein: "… God does not play dice with the universe!", any deepening of the knowledge of the F_1F_O-complex confirms it as an example of intelligent rotary design that respects the thermodynamic rules of rotation in a nano-universe. Additionally, the chirality of *a* subunit may have implications not only for mitochondrial and bacterial F_1F_O-complexes but also for other structurally related F-ATP synthases.

Unfortunately, as far as we are aware, the molecular machinery within the membrane portion F_O is much less clear than that of the catalytic portion F_1. Up to now the molecular structure of *a* subunit has not been experimentally defined and studies should necessarily be addressed to unravel it. Hopefully, the experimental identification of all steps of proton translocation within a subunit will move our knowledge beyond the hypothesis.

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