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TOPICAL REVIEW

The *c*-Ring of the F_1F_O-ATP Synthase: Facts and Perspectives

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Abstract The F_1F_O-ATP synthase is the only enzyme in nature endowed with bi-functional catalytic mechanism of synthesis and hydrolysis of ATP. The enzyme functions, not only confined to energy transduction, are tied to three intrinsic features of the annular arrangement of *c* subunits which constitutes the so-called *c*-ring, the core of the membrane-embedded F_O domain: (i) the *c*-ring constitution is linked to the number of ions (H^+ or Na^+) channeled across the membrane during the dissipation of the transmembrane electrochemical gradient, which in turn determines the species-specific bioenergetic cost of ATP, the "molecular currency unit" of energy transfer in all living beings; (ii) the *c*-ring is increasingly involved in the mitochondrial permeability transition, an event linked to cell death and to most mitochondrial dysfunctions; (iii) the *c* subunit species-specific amino acid sequence and susceptibility to post-translational modifications can address antibacterial drug design according to the model of enzyme inhibitors which target the *c* subunits. Therefore, the simple *c*-ring structure not only allows the F_1F_O-ATP synthase to perform the two opposite tasks of molecular machine of cell life and death, but it also amplifies the enzyme's potential role as a drug target.

Keywords F_1F_O-ATP synthase · *c*-Ring, mitochondria · Bioenergetic cost · Drug-binding region · Mitochondrial permeability transition

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Introduction

The membrane-bound ATP synthase or F_1F_O-ATPase (EC 3.6.3.14) has the primary role of synthesizing ATP, the molecular energy currency of living cells. Its catalytic ability coupled to a nearly ubiquitous occurrence makes it deserve the universal definition of enzyme of life (Junge and Müller 2011). Its prominent role in the bioenergetic machinery of the cell is equaled by a fascinating nano-molecular architecture which enables the F_1F_O complex to work as a frictionless engine (von Ballmoos et al. 2008), by a key molecular strategy, based on the so-called torque generation (Junge et al. 2009). Accordingly, the energy transfer within the enzyme complex is based on a torsional mechanism which matches the work of two distinct enzyme sectors of the F_1F_O-ATP synthase: the extrinsic sector or F_1, which protrudes outside of the membrane and catalyzes the synthesis/hydrolysis of ATP, and the transmembrane sector or F_O, which channels ions across the membrane (Walker 2013). The two opposite functions of ATP synthesis and hydrolysis are based on the capability of the membrane-embedded rotor to rotate in two directions. In the presence of transmembrane proton motive force (Δp), protons flow downhill across the membrane. This proton translocation makes the central stalk, which connects F_O to F_1, rotate counterclockwise (viewed from F_1). Such rotation is promptly transmitted to the catalytic sector F_1 that undergoes conformational changes which allow the synthesis of ATP from ADP and P_i. Basically, the whole enzyme machinery is based on a chemomechanical coupling which implies a Δp-driven rotation of the molecular motor F_O at about 130 Hz (Nicholls and Ferguson 2013). Conversely, if the membrane is not enough energized, in other words if Δp is poor, the same F_1F_O complex acts as ATPase, namely it hydrolyzes ATP to ADP and P_i. In this

case, the energy obtained from ATP hydrolysis is exploited to move protons uphill across the membrane through a clockwise rotation (viewed from F_1) of F_O, leading to Δp re-building (Dimroth et al. 2006). The thermodynamic equilibrium between the phosphorylation potential (ΔG_p) of ADP and Δp determines which of the two opposite processes of synthesis/hydrolysis of ATP occurs. In other words, if Δp prevails on ΔG_p, ATP is synthesized and if ΔG_p prevails, ATP is hydrolyzed. The mechanism of proton translocation within F_O is not yet fully understood. The fundamental constituents of the torque generation are the *a* subunit and the *c*-ring. The nature of *a* subunit is still partially obscure and controversial. Within *a* subunit, two aqueous ion semi-channels (Steed and Fillingame 2008) span across the membrane from the matrix to the intermembrane space. Unexpectedly, recent advances suggest that the semi-channel on the matrix side has horizontal helices, while the semi-channel at the luminal side would be vertical (Allegretti et al. 2015), namely in the same direction as proton flux. A quite complicated mechanism has been depicted (Elston et al. 1998; Stock et al. 1999), somehow based on the asymmetry of the two aqueous semi-channels within *a* subunit (Nesci et al. 2015) and on the properties of the so-called *c*-ring, a membrane-embedded cylindrical rotor which allows the flux of protons across the membrane and is the master player in both ATP synthesis and hydrolysis (Nicholls and Ferguson 2013).

The size and the constitution of the *c*-ring, conserved in the same species and apparently linked to the species bioenergetic requirement (von Ballmoos et al. 2008; Nesci et al. 2013a), determine the number of protons translocated by a complete (360°) rotation of the rotor to synthesize ATP and impose the bioenergetic cost of ATP to the species (Watt et al. 2010). The *c*-ring's role in the bioenergetic cost of ATP is universal, being the Δp-driven rotary mechanism of the F_O rotor shared by prokaryotes and eukaryotes.

Recent advances also unravel the involvement of the F_1F_O complex, and especially of the *c*-ring, in cell death. Accordingly, due to the enzyme capability of switching the energy transduction system from the energy-saving to the energy-dissipating mode, the F_1F_O-ATPase has been involved in the crucial event of the mitochondrial permeability transition (MPT), which triggers cell death (Bernardi et al. 2015). In turn, MPT has been related to the opening of the mitochondrial permeability transition pore (MPTP) in which the *c*-ring has been hypothetically involved. Once identified, the molecular components of MPTP can be exploited as strategic targets to treat pathologies related to MPT such as neurodegenerative disorders, cardiovascular diseases, and cancer (Bernardi and Di Lisa 2015; Bonora et al. 2015). Consistently, the F_1F_O complex, and specifically the *c*-ring, raises increasing interest in pharmacology (Sakthivel 2012).

Thus, in recent years, "the ATP synthase—a splendid molecular machine" (Boyer 1997), extensively studied in past decades for its key energy-saving role, turns out as an amazing multi-tasking enzyme. Its roles span from life to death with increasing corollary implications not only in the so-called mitochondrial diseases, but also, quite unexpectedly, in a wide variety of common pathologies. Among the enzyme constituents, the membrane-embedded *c*-ring emerges not only as a crucial structure in both catalytic functions of the enzyme, but also as one of the preferred sites of action of modulators and modifiers.

ATP Cost and *c*-Ring Size: The Structural Basis of Bioenergetics

The *c*-ring is the essential component of the F_O rotor. In all organisms, it is constituted by monomers (*c* subunits) arranged in a circle as a cylindrical palisade which form and contain an internal phospholipid-containing hydrophobic cavity (Oberfeld et al. 2006). As far as we are aware, this universal *c*-ring constitution has few exceptions, as we will see later. Each hydrophobic hairpin-shaped *c* subunit constitutes the basic unit of the ring: the N- and C-terminal α-helices of each subunit flank the same terminal regions of another subunit thus forming two concentric circles. In eukaryotes, these two circles in each *c* subunit are linked by a loop region which faces the head-group region of phospholipids on the matrix side of the inner mitochondrial membrane (Walker 2013). The *c*-ring is directly involved in the proton translocation across the membrane which is based on its structural properties. According to the depicted molecular model, the protons to be translocated flow within the semi-channel facing the positive side of the bilayer and protonate a carboxyl group, Asp or Glu depending on the species, at approximately half of the transmembrane C-terminal α-helix of a *c* subunit. The protonated carboxyl group switches from the open conformation to the locked conformation, which is energetically favored to penetrate into the membrane during the *c*-ring rotation. In this way, the protonated *c* subunit, namely in its neutral form, reaches the semi-channel of the *a* subunit facing the negative membrane side. Then the carboxylic group returns to the open conformation being its deprotonation favored by the basic environment (Pogoryelov et al. 2009, 2010; Symersky et al. 2012a).

Interestingly, the *c*-ring stoichiometry is constant in a given species, but the number of *c* subunits can be in the range 8–15 and consequently the *c*-ring size differs among species (von Ballmoos et al. 2009) (Fig. 1). Eukaryotic ATP synthases have small *c*-rings, while prokaryotic and chloroplast enzymes usually contain large *c*-rings, with up to 15 subunits in cyanobacteria (Pogoryelov et al. 2005).

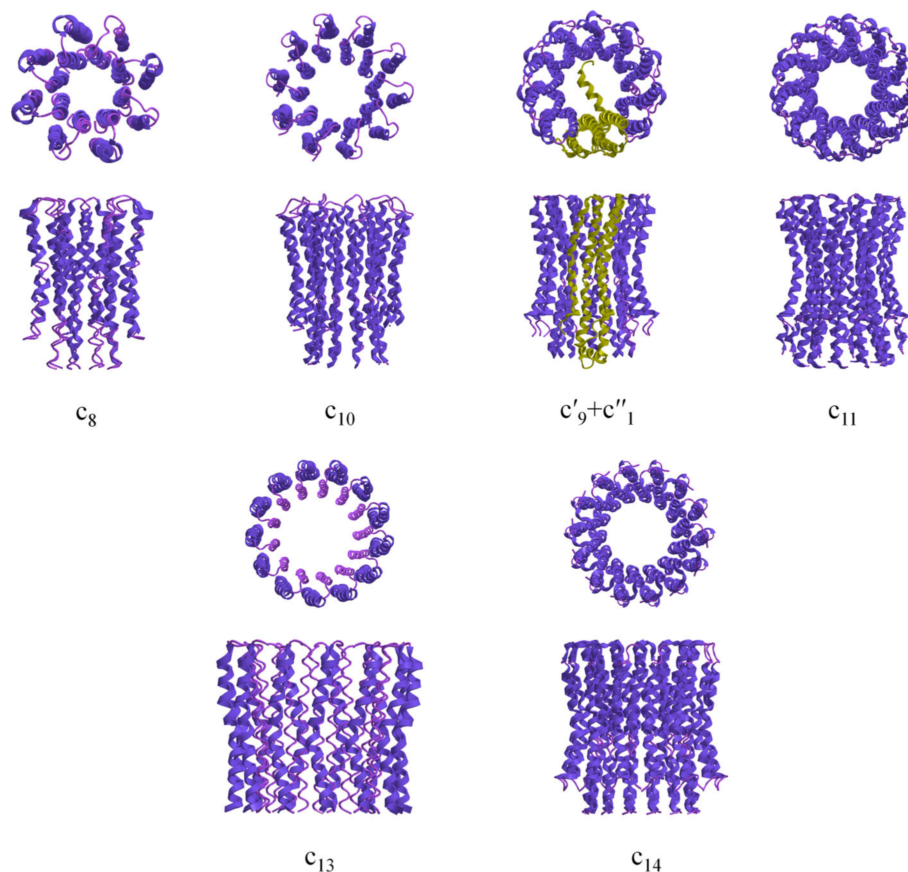

Fig. 1 Comparison between the *c*-ring structures of different organisms. Crystal structures of *c*-rings for F-type ATP synthases: *Bos Taurus*, 8 *c* subunits, PDB accession no. 2XND (Watt et al. 2010); the yeast *Saccharomyces cerevisiae*, 10 *c* subunits, PDB accession no. 3ZRY (Giraud et al. 2012); the bacteria *Ilyobacter tartaricus,* 11 *c* subunits, PDB accession no. 1YCE (Meier et al. 2005); *Acetobacterium woodii,* non-homomeric 9 + 1 (the double-hairpin *c* subunit is in gold), accession no. PDB 4BEM (Matthies et al. 2014); *Bacillus pseudofirmus,* OF4 13 *c* subunits, accession no. PDB 2X2V (Preiss et al. 2010); and the chloroplast *Spinacia oleracea* 14 *c* subunits, accession no. PDB 2W5J (Vollmar et al. 2009). These differently sized *c*-ring structures bind Na^+ (*I. tartaricus and A. woodii*) or H^+ (*B. taurus*, *S. cerevisiae*, *B. pseudofirmus*, and *S. oleracea*). Each *c*-ring structure is viewed from the *top* and laterally to illustrate the spatial arrangement of the *c* subunits

Probably, the different size of the *c*-ring may result from evolutionary adaptation to the various environmental or cellular conditions of ATP production (von Ballmoos et al. 2008). In eukaryotes, the inner helix of each *c* subunit accommodates the highly conserved motif of four glycine residues (G × G × G × G), which confers tight transmembrane α-helix packing, without space for side chains (Vonck et al. 2002). Interestingly, under conditions of low overall Δp in alkaline environment (e.g., in the extreme alkaliphilic *Bacillus pseudofirmus* OF4), the G × G × G × G motif is replaced by A × A × A × A, namely the methyl side chain of alanine replaces the hydrogen of glycine. This amino acid substitution enhances both the *c*-ring stoichiometry and its size by stretching the encoded amino acid sequence at the N-terminal helix (Preiss et al. 2013). In most cases, according to the mechanism of proton transport depicted above, the number of *c* subunits is tightly linked to the number of transported protons. However any rule can have exceptions. The F/V-hybrid rotor ring, which from an evolutionary standpoint lies between the ATP synthase and H^+ pumps, is a good example of a condition in which the number of ion-binding sites which establishes the number of transported protons does not correspond to the number of *c*-ring subunits. In *Acetobacterium woodii*, the heteromeric architecture of the *c*-ring (9:1 of F- and V-type *c* subunits, respectively) contains single-hairpin *c* subunits which host one ion-binding site and a double-hairpin *c* subunit with one additional site and one vacancy which does not bind ions (Matthies et al. 2014). Apparently, during evolution, living organisms have adapted the *c*-ring size to work optimally under the conditions dictated by the prevailing electrochemical parameter (Nesci et al. 2013a). Accordingly, Δp consists of two components: the electric gradient, namely the

transmembrane membrane electric potential (Δφ), and the chemical gradient, namely the difference in proton concentrations between the two sides of the membrane (ΔpH).

When Δφ is the dominant driving force (e.g., in mitochondria), the *c*-ring is small. Conversely, in chloroplasts, where a large ΔpH is accompanied by a small Δφ, the F_1F_O-ATPase has a large *c*-ring (e.g., formed by 14 subunits, as shown in Fig. 1) to compensate for the low Δφ (von Ballmoos et al. 2008). This functional adaptation is somehow explained by a mechanical analogy on considering how the gear on the back wheel of a bicycle works (Nicholls and Ferguson 2013). A small gear requires a lot of force, but few rides, to make the back wheel rotate. Thus, the rotation of the small mitochondrial *c*-ring requires the translocation of a less number of protons across the membrane, being proton flux driven by the high Δφ. On the other hand, large *c*-rings can rotate in the presence of low Δφ and high ΔpH by translocating a high number of protons (von Ballmoos et al. 2008; Nesci et al. 2015).

The rotation angle of the rotor is inversely proportional to the number of *c* subunits (*n*). Indeed, in a complete rotation of the rotor (360°), a low *n* value (i.e., small *c*-ring) implies a higher rotation angle, for each *c* subunit, than a high *n* value (i.e., large *c*-ring). The translocation of the same number of protons by differently sized *c*-rings can be only obtained if small *c*-rings perform more revolutions than large *c*-rings. In other words, since each 360° rotation means three synthesized ATPs, from a bioenergetic standpoint small *c*-rings have higher return than large *c*-rings, since they produce more ATP molecules for a stated number of translocated protons. In general, since each F_O-*c* subunit possesses a protonation site, during a 360° rotation of the rotor the protons are translocated across the membrane. At the same time, since the F_1 sector has three substrate-binding sites (one on each β-subunit) (Boyer 1997), the synthesis or the hydrolysis of 3 ATP molecules occurs, according to the direction of rotation of the rotor. The thermodynamic parameter H^+/ATP ratio (*i*) defines the energy cost of ATP synthesis by the F_1F_O-ATPase. Accordingly, the stoichiometric ratio of *c* subunits to β-subunits also coincides with *i* in F-type ATPase (Petersen et al. 2012). The bioenergetic cost of ATP is linked to the number of ATP molecules synthesized for each electron pair transferred from a defined substrate (NADH or succinate) via the respiratory complexes to oxygen (Ferguson 2010). The relationship between phosphorylation and respiration/oxidation is simplified by the P/O ratio or $P/2e^-$ ratio. The consensus values for the number of protons pumped out in the intermembrane space per electron pair transferred to O_2 (H^+/O) are 10 protons for NADH and 6 protons for succinate as substrates, and these values are constant. Therefore, the number of synthesized ATP molecules for each oxidized substrate can be calculated from the ratio of respiration stoichiometry (H^+/O) to *i*. However, the transmembrane transport of ADP and phosphate (P_i), required to form ATP, namely the electrogenic $ATP^{4-}_{(out)}/ADP^{3-}_{(in)}$ antiport by the adenine nucleotide translocator (ANT) and the electroneutral H_2PO^{4-}/H^+ symporter or H_2PO^{4-}/OH^- antiporter by the phosphate carrier (P_iC), should be considered. Therefore, the denominator of the fraction which defines the P/O ratio is $i + 1$:

$$P/O = \frac{H^+/O}{i+1}. \quad (1)$$

Even if the P/O ratio is a mechanistic constant for any particular substrate (Brand and Nicholls 2011), it depends on the *c*-ring size, which is directly linked to *i*. Accordingly, at constant H^+/O ratio, a small *c*-ring builds more ATP molecules than a large *c*-ring (Silverstein 2014), since the low *n* value linked to *i* decreases the denominator of the fraction and increases the P/O ratio.

The ΔG_p and Δp values for the F_1F_O-ATP synthase are mutually related by the thermodynamic relationship:

$$\Delta G_p = (i+1)F\Delta p, \quad (2)$$

in which *i* is a variable and *F* is the Faraday constant. At fixed ΔG_p, Δp and ($i + 1$) are inversely proportional. As a corollary, in mammalian mitochondria to generate a given ΔG_p in the presence of higher Δp, a lower value of *i* is required with respect to other organisms (Nicholls and Ferguson 2013). This condition should be fulfilled to yield ATP synthesis, since the F_1F_O-ATPase switches between the two opposite modes of ATP hydrolysis/synthesis according to the reversal potential threshold (E_{rev}), which in turn is inversely proportional. In other words, E_{rev} increases as *i* decreases. This implies that in mitochondria the F_1F_O-ATPase is especially sensitive to a potential drop, namely a slight membrane depolarization will trigger the transition from ATP synthesis to ATP hydrolysis (Nesci et al. 2015). To sum up, a small *c*-ring requires only few protons to produce a single molecule of ATP but, as a consequence, it requires high Δp to allow ATP synthesis.

Therefore, the bioenergetic cost of ATP, the energy currency of the cell, is related in a very simple way with the *c*-ring stoichiometry, which finely tunes the thermodynamic parameters of oxidative phosphorylation.

The *c*-Ring and the Mystery of MPT

Recent advances increasingly hint that the ATP synthase, and especially the *c*-ring, may constitute or contribute to the formation of the mysterious MPTP, a proteinaceous pore in the inner mitochondrial membrane, whose

molecular architecture is still an enigma. In turn, MPTP is the key element of the MPT, the so-called mitochondrial permeability transition, first discovered in 1970s (Hunter and Haworth 1979), which dramatically changes the permeability features of the mitochondrial membrane. Alterations in the mitochondrial inner membrane permeability to ions and solutes, with molecular masses up to approximately 1.5 kDa, lead to matrix swelling. The MTP, which could be due to the reversible opening of the MPTP, commits cells to suicide via regulated necrosis or apoptosis (Bernardi et al. 2006). The MPT is triggered by high Ca^{2+} concentrations in mitochondria, stimulated by P_i and certain fatty acids, sensitized by oxidative stress, and conversely inhibited by Mg^{2+} and ADP (Lehninger 1959; Azzone and Azzi 1965a, b). A crucial role in MPT modulation was assigned to cyclosporine A (CsA), in turn pharmacological target of cyclophilin D (CyP-D), also known to be relevant in MPTP regulation (Crompton et al. 1988).

In search for the structural bases of MPTP, several models have been hypothesized. According to a first model, never experimentally confirmed, the outer and inner mitochondrial membranes would generate a contiguous pore constituted by the voltage-dependent anion channel (VDAC) embedded in the outer mitochondrial membrane (Baines et al. 2007) and by the ANT in the inner mitochondrial membrane (Kokoszka et al. 2004). On the other hand, recent evidence suggests that the two outer and inner mitochondrial membranes work separately to open the MPTP. The Bcl-2 protein family members Bax and Bak would facilitate the permeability of the outer membrane involved in MPTP (Karch et al. 2013), while the inner component, whose molecular entity remains up to now elusive, would constitute the regulatory site. After about 60 years of studies on the nature of the inner pore-forming membrane, the MPTP was found to generate currents with indistinguishable features from those produced by the Ca^{2+}-dependent F_1F_O-ATPase dimers (Antoniel et al. 2014). The H^+-pumping activity of F_1F_O-ATPases could be different if Ca^{2+} or Mg^{2+} acts as a cofactor. Indeed, ATP hydrolysis stimulated by Ca^{2+} was uncoupled to proton pumping in beef heart submitochondrial particles (Papageorgiou et al. 1998); similarly Ca^{2+}-dependent F_1F_O activities uncoupled to proton pumping were detected in *Rhodospirillum rubrum* (Nathanson and Gromet-Elhanan 2000). Perhaps, the different ligand (Ca^{2+} or Mg^{2+}) may promote a different conformational state of the catalytic site. Changes in the interactions between the β and γ subunits of F_1 are known to be required to link catalysis to proton translocation through F_O (Nathanson and Gromet-Elhanan 2000). This link (namely the coupling) is known to be signaled by the ATPase sensitivity to the specific inhibitor oligomycin (Runswick et al. 2013). Reconstituted mitochondrial F_1F_O-ATPase dimers treated with Ca^{2+} create a MPTP-like activated channel. In this structure, CyP-D in the presence of P_i inhibits the F_1F_O-ATP synthase activity by binding to the OSCP subunit (Giorgio et al. 2009), while CsA removes CyP-D and reactivates the enzyme. Interestingly, in recent years a prominent role of the F_1F_O-ATPase c-ring in MPTP constitution is emerging (Bonora et al. 2015) since agents targeting or gene-silencing the c subunits protect against MPTP opening (Bonora et al. 2013). Moreover, the phosphorylation status of c subunits might modulate the MPTP kinetics and cation selectivity (Azarashvili et al. 2014). Consistently, the c-ring was shown to generate a non-specific current ascribable to the MPTP (Alavian et al. 2014). However, the newly proposed mechanisms involving the c subunits reconstituted in membranes in the formation of a pore refractory to CyP-D are still unclear. Assumed that MPTP opening caused by Ca^{2+} overload in mitochondria would detach the c-ring from F_1, CsA and other MPTP inhibitors would prevent this decoupling (Alavian et al. 2014). However, the intriguing putative role of the c-ring in MPTP has not been confirmed yet. The findings up to now reported on this topic appear in some cases contradictory and do not justify the MPTP regulation by its effectors. The finding of Bernardi's group that only purified F_1F_O-ATPase dimers, and not monomers, have MPTP-like channel activity (Giorgio et al. 2013), once confirmed, will open new frontiers in the identification of the F_1F_O-ATPase portion involved in MPTP formation. However, the known features of the inhibitory factor 1 (IF_1), which promotes F_1F_O-ATPase dimerization, supports *cristae* structure, preserves the mitochondrial morphology and ultrastructure, protects against the apoptotic/necrotic death linked to the ATPase inhibition (Faccenda et al. 2013), and apparently weakens the putative involvement of ATP synthase dimers in MPTP formation.

Other findings support the hypothesis that the c-ring may associate with other membrane proteins such as ANT and P_iC, at first considered as MPTP components, to form the ATP synthasome complex (Chen et al. 2004). In such complex, ANT and P_iC could modulate the pore-forming capability of the c-ring (Halestrap 2014).

To sum up, the whole matter is still controversial, and up to now contradictory evidence cannot lead to define which component of the ATP synthase is involved in the MPTP. Indeed, if the putative F_1F_O-ATPase role in the mysterious MPTP constitution will be confirmed, the F_1F_O complex, and especially its master player c-ring, will emerge as a prominent multi-subunit protein of both life and death.

The Common Drug-Binding Site on the *c*-Ring and the Modulation of Drug Sensitivity

Apart from the presumptive involvement of the *c*-ring in MPTP, the F_1F_O complex is increasingly believed as a molecular switch between life and death. Accordingly, the assertion "no ATP synthase no life" is much more than a slogan (Nesci et al. 2014a). Due to its double-faced role, the enzyme complex can be effectively exploited to regulate energy metabolism and selectively fate cells to death. Thus, it emerges as an ideal molecular target for drugs (Sakthivel 2012). Among the wealth and wide variety of F_1F_O-ATPase inhibitors, most compounds were initially shown to act on the catalytic activity of the hydrophilic domain of F_1, the much more studied sector. Interestingly, other compounds do not affect F_1, at least at low concentrations, and prevent the proton translocation through the embedded F_O domain (Hong and Pedersen 2008) by specifically binding to *c* subunits. Often these compounds, due to this molecular mechanism, block both enzyme functions, namely ATP synthesis and ATP hydrolysis. Additionally, the *c*-ring was shown to embrace a common drug-binding region in which different compounds can also localize simultaneously by selectively interacting with their specific binding sites and display different inhibitory potency (Nesci et al. 2014c).

The amino acid sequences of *c* subunits are identical for almost all vertebrates and highly conserved in invertebrates (Watt et al. 2010). However, they significantly vary between eukaryotes and prokaryotes and even among different bacterial species. Since the structure and functionality of the *c* subunits in F_1F_O-ATPase are universal, the identification and isolation of drugs selectively acting on the bacterial enzyme is a crucial challenge. Accordingly, renewed hope for innovative therapies against recalcitrant diseases sustained by bacteria might stem from the exploitation of slight differences between eukaryotic and prokaryotic proteins which can serve as molecular tools to selectively manipulate the F_1F_O-ATP synthase function. Differently from other bacteria, mycobacteria entirely rely on the ATP synthase for optimal growth (Singh et al. 2013). Diarylquinolines, which target F_1F_O-ATPase *c* subunits of *Mycobacterium tuberculosis* (Koul et al. 2007), are an emerging class of antimycobacterial drugs under clinical trials. Among them, bedaquiline, also known as TMC207 or R207910, has high selectivity for the mycobacterial enzyme, which displays a more than 20,000-fold higher affinity for the drug than the mitochondrial F_1F_O-ATPase (Haagsma et al. 2009). This property minimizes or even rules out any concern of possible toxic side effects on the host eukaryotic enzyme and encourages its clinical use. The drug efficiently inhibits the proton translocation by binding to the *c*-ring of mycobacteria (Preiss et al. 2015), favored by the appropriate amino acid sequence of *c* subunits of *M. tuberculosis*. Bedaquiline acts on all life stages of the microorganism (Lakshamanan and Xavier 2013), probably because the species-specific *c*-ring composition is maintained. Indeed, the crystal structure of c_9-ring from non-pathogenic *M. phley* shares a tight sequence identity with *M. tuberculosis*, especially in the transmembrane portion of *c* subunits which binds bedaquiline (Andries et al. 2005; Haagsma et al. 2009). The H^+-binding site, in each *c* subunit, is provided by glutamate (E61) on the C-terminal α-helix. The oxygen atoms of E61 carboxyl group are engaged in a hydrogen bond network with D28 on the N-terminus of the same α-helix, which stabilizes the glutamate conformation. A second bond formed with one water molecule coordinates the oxygen of backbone carbonyl of L59 on the C-terminus of the adjacent monomer. In detail, the amino acid side chains of the ion coordination region, as well as I66 from the adjacent C-terminal α-helix, define a cleft motif between two *c* subunits which anchor the diarylquinoline molecule (Segala et al. 2012). The complex net built by the different interactions which tightly anchor bedaquiline to *c* subunits has been recently defined (Preiss et al. 2015) (Fig. 2). The overall interaction pattern is very similar in *M. tuberculosis* and *M. phley*, even if the position of the amino acid residues involved is slightly different in the two species, as detailed below. In both cases, a wide spectrum of interactions is established between bedaquiline and the surrounding amino acid residues of the C-terminal α-helices of two *c* subunits, i.e., the protomer *c*H, which contains the proton-binding site blocked by the drug, and the adjacent protomer *c*A. A close insight reveals an intriguing net of multiple interactions which are summarized as follows. The quinolone moiety of the drug binds to the residues G58 (G62 in *M. phley*), E61 (E65 in *M. phley*), A62 (A66 in *M. phley*), and F65 (F69 in *M. phley*) of *c*H. In addition, E61 (E65 in *M. phley*) of *c*H interacts through a specific hydrogen bond with the dimethylamino moiety of the drug which penetrates between the two adjacent monomers. Furthermore, bedaquiline through its hydroxyl group establishes water molecule bridges between the oxygen of backbone carbonyl and the carboxyl group of E61. The dimethylamino moiety of bedaquiline is also bound with Y64 (Y68 in *M. phley*) of *c*H, and with A62 (A66 in *M. phley*) and A63 (A67 in *M. phley*) of *c*A, while the naphthyl group establishes hydrophobic interactions among Y64 (Y68 in *M. phley*) and L68 (L72 in *M. phley*) of *c*H and I66 (I70 in *M. phley*) of *c*A. Finally, F65 (F69 in *M. phley*) of *c*H, as a result of a conformational change induced by binding to the quinolone moiety, creates van der Waals interactions

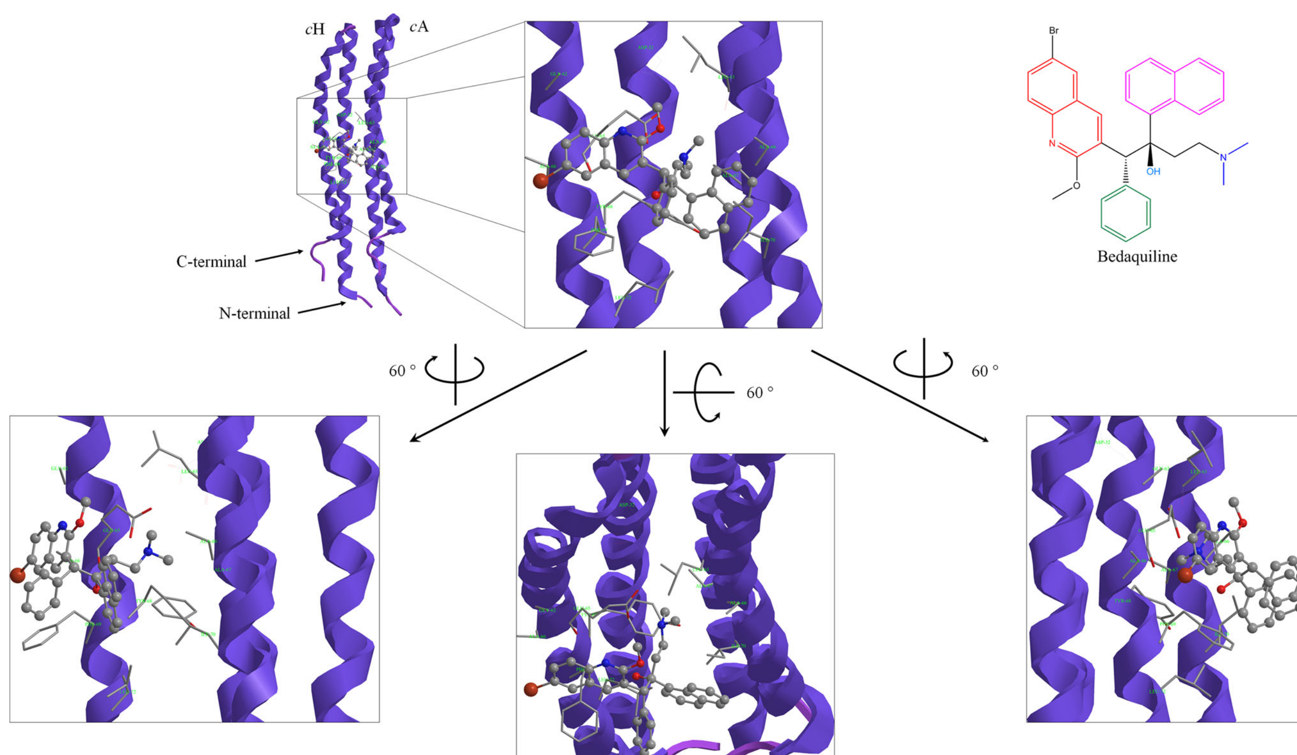

Fig. 2 Putative involvement of different amino acids of two adjacent c subunits (cH and cA) in bedaquiline binding according to recent literature (Preiss et al. 2015). The two c subunits cH and cA are drawn in magenta. The H^+-binding site characterized by the conserved carboxylate of E65 is engaged in a network of electrostatic bonds with the amino acid residues shown as wire frame (as detailed in the text). The side chains of amino acids which specifically interact with the bedaquiline molecule (as ball-and-stick model) are drawn as stick models. The conformation of F69 is illustrated irrespective of bedaquiline binding. The bond between bedaquiline and c subunits was generated according to PDB ID: 4V1F. Bedaquiline structure is illustrated on the *top right corner*: in *red* the quinolone ring, in *pink* the naphthyl residue, in *blue* the dimethylamino moiety, in *green* the phenyl moieties, and in *sky blue* the hydroxyl group. All these groups are involved in bedaquiline binding to c subunits (Color figure online)

with the bedaquiline phenyl group (Preiss et al. 2015) (Fig. 2). The variety and the abundance of specific molecular interactions, which cannot be established in other prokaryotes and in eukaryotes, concur to determine the high affinity of the mycobacterial c-ring for bedaquiline. Moreover, the proton barrier of the guanidinium group of arginine (R) of the a subunit (Mitome et al. 2010) acts on the ion-binding site during the putative intermediate rotation states of the c-ring (Pogoryelov et al. 2009). The arginine interaction is somehow mimicked by the intermolecular bond between E61 and dimethylamino moiety of bedaquiline in the membrane surrounding the c-ring. This bond would act as a trap in H^+ transport mechanism of F_O. Accordingly, the drug bound to the c-ring cannot attain the tight polar a/c interface of the F_1F_O-ATPase. According to Meier's group (Preiss et al. 2015), only a single molecule of bedaquiline completely blocks the F_1F_O-ATPase torque generation. Therefore, any substitution in the amino acid which defines the cleft motif between two adjacent c subunits will produce bedaquiline-resistant mutants of *M. tuberculosis* (Segala et al. 2012).

If up to now the genus *Mycobacterium* emerges as the only suitable target of bedaquiline, in recent years novel diarylquinolines, differing from bedaquiline in the side chains and also targeting bacterial c subunits, emerged as promising drugs to fight resistant strains of Gram-positive bacteria, including *Staphylococcus aureus* and *Streptococcus pneumoniae* (Balemans et al. 2012). It seems likely that the antibacterial spectrum of diarylquinolines can be even broadened, maintaining the same molecular target, by providing slight changes in their molecular structure (Singh et al. 2014) to optimize their selective binding to the c-ring.

The interaction of macrolide antibiotics with F_O and specifically with the c-ring are an emerging field of study. Oligomycin, a specific inhibitor of transmembrane F_O domain (which takes the O subscript from oligomycin), as well as other macrolide antibiotics, inhibits the H^+ translocation required for the torque generation, in turn essential for the F_1F_O-ATPase machinery (Nesci et al. 2014b). Oligomycin belongs to the polyketide class of macrolide antibiotics whose basic structure consists of polymers of ketide units arranged to build a large lactone

ring covalently bound to one or more deoxy sugars. The macrolide ring confers the inhibition power, while the deoxy sugar moiety is not crucial for the ATPase inhibition (Salomon et al. 2001). The oligomycin-binding site on the *c* subunits has been defined at high-resolution (1.9 Å) crystal structure (Symersky et al. 2012b). Interestingly, macrolide drugs featured by different polyketide backbones display a similar inhibition mechanism but a different inhibition potency. Oligomycin binds to the amino acid side chains of two adjacent *c* subunits of F_O, *c*1 and *c*2, and covers the H^+-binding site (E59) on *c*1. By shielding the carboxyl access to the aqueous environment of the proton half channel of *a* subunit, oligomycin blocks the proton flux within F_O without affecting the *c*-ring backbone conformation (Symersky et al. 2012b). On the contrary, on the C-terminal α-helices the side chains of L63 of *c*1 subunit and F64 of *c*2 subunit would rotate to allow the propanol residue linked to the spiropyranose sugar rings of oligomycin to penetrate between the two *c* monomers so as to establish non-covalent interactions with the α-helical N-terminus of the *c*1 subunit. The macrocyclic lactone ring is involved in both hydrophilic and hydrophobic interactions with 7 amino acids of the C-terminal α-helices of *c1* subunit and 5 amino acids of the C-terminal α-helix of *c*2 subunit. The proton-binding site (E59 of *c1* subunit) is also involved in oligomycin binding via a bridging water molecule (Symersky et al. 2012b). Multiple inhibition analysis of the F_1F_O-ATPase carried out with different macrolides slightly differing in the polyketide ring pointed out that each compound requires a different amino acid combination to bind to the *c*-ring (Nesci et al. 2014c). Therefore, the decrease in the mitochondrial ATPase sensitivity to macrolides produced by post-translation modification (Pagliarani et al. 2013) and the multiple drug resistance induced by amino acid mutations (Galanis et al. 1989; Nagley et al. 1986) are consistent in strongly suggesting that macrolide antibiotics share a common binding region within F_O in which each compound specifically binds to its specific site (Nesci et al. 2014c).

All mitochondrial *c* subunits have a conserved cysteine on the α-helix C-terminal. Interestingly, post-translational modifications at this conserved cysteine can substantially modify the common drug-binding site. The same sensitivity loss to oligomycin induced by thiol oxidation was found in different animal phyla (Nesci et al. 2011, 2014b). The F_1F_O-ATPase desensitization to oligomycin can be

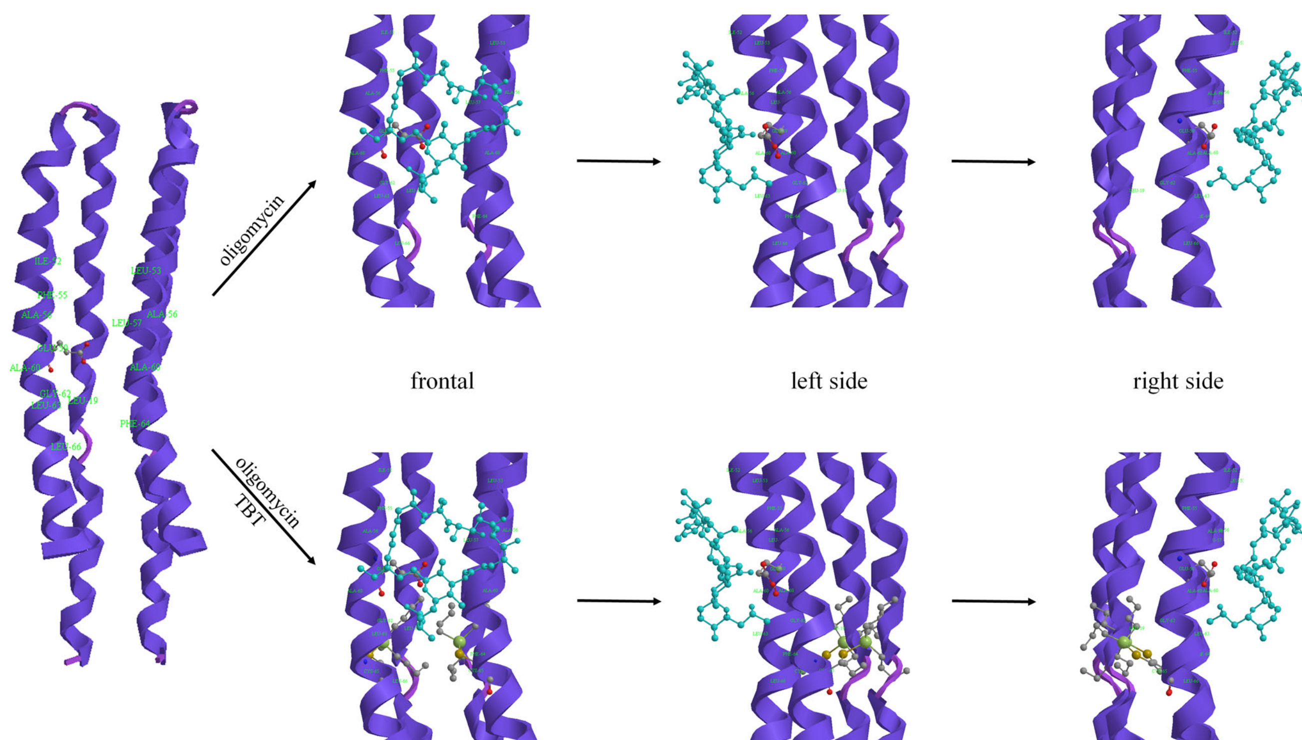

Fig. 3 TBT binding drives the oligomycin desensitization by modifying the *c* subunits. Oligomycin binding to two adjacent *c* subunits of *S. cerevisiae* (*upper panel*) and model of the F_1F_O-ATPase oligomycin sensitivity loss due to TBT binding to cysteines' thiols (*lower panel*). The model of oligomycin binding to *c* subunits (in *violet*) was generated according to PDB ID: 4F4S (Symersky et al. 2012b). In the illustration on the left, the amino acid acronyms highlighted in green indicate the residues which interact with oligomycin. The carboxylic side chain (carbon in *gray* and oxygen in *red*) of the proton-binding site, oligomycin (in *sky blue*), and TBT, in which tin (in *green*) with the four butyl chains (in *gray*) is bound to cysteine sulfur (in *yellow*), are drawn as ball-and-stick models (Color figure online)

extended to all inhibitors which share the binding region on the enzyme. In contrast, the *c* subunits of prokaryotic ATP synthases lack cysteine on the external transmembrane α-helix, and this crucial site is refractory to post-translational modifications involving cysteines (Nesci et al. 2014b). Indeed, in *Saccharomyces cerevisiae* strains, a single amino acid mutation (Cys65Ser) in the C-terminal α-helix of *c* subunit induces oligomycin resistance (Sebald et al. 1979). The mitochondrial F_1F_O-ATPase desensitization to macrolide drugs by tributyltin (TBT) is due to TBT binding to a low-affinity site on F_O (Nesci et al. 2012), and this effect was not produced by other butyltins (Nesci et al. 2013b). Accordingly, when oligomycin blocks the rotation of the *c*-ring, TBT binding, by reversibly oxidizing critical thiols on the *c*-ring, may promote a conformational change in the oligomycin–enzyme complex, which in turn would destabilize the drug-binding site (Fig. 3) allowing proton translocation recovery (Nesci et al. 2014c). Therefore, the F_1F_O-ATPase susceptibility to macrolide antibiotics can be abolished in eukaryotes and maintained in bacteria, due to the different amino acid sequences of *c* subunits and particularly to the lack of the crucial cysteines in bacteria.

Thus, the structural features of the *c*-ring could *per se* allow the molecular switch off to drugs of the F_1F_O-ATPase. Differences in the amino acid sequence between eukaryotic and prokaryotic *c* subunits can be exploited to specifically inhibit proton translocation in one species without affecting that in another species (Nesci et al. 2014a). On the other hand, structural studies on post-translational modifications of F_1F_O-ATPase *c* subunits can elicit structure-related changes in the protein functionality which may be exploited in therapy. According to a fascinating perspective, the *c*-ring could be made insensitive to drugs in eukaryotes by post-translational modifications, while, at the same time, the different amino acid composition in the bacterial enzyme counterpart would make the *c*-ring refractory to these chemical modifications and preserve the sensitivity to the drug. Clearly, the ideal antimicrobic drug kills bacteria without eliciting significant side effects in the eukaryotic host. On these bases, the possible modulation of the *c*-ring structural properties opens intriguing perspectives in pharmacology and stimulates the development of new drugs targeting the ATP synthase and specifically the *c*-ring.

Conclusion

The *c*-ring of the F_1F_O-ATP synthase is the amazing demonstration of how a simple polymeric structure may sustain multiple key functions. Accordingly, the *c*-ring plays a crucial role in cellular bioenergetics as its constitution is somehow adaptive to the metabolic requirements of the species. The unique function of thermodynamic machine of the F_1F_O-ATPase in oxidative phosphorylation, which *per se* is extraordinary in biology, would be extended if the *c*-ring's role in the MPT will be fully validated. In addition, the *c*-ring, as a target of different drugs, could enhance the therapeutic potential of the mitochondrial enzyme and open the door to new medical approaches.

To sum up, indeed we can say that the F_1F_O-ATP synthase is "the lord of the (*c*-)ring."

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