Mechanism of the F_1F_0-type ATP synthase, a biological rotary motor

Roderick A. Capaldi and Robert Aggeler

The F_1F_0-type ATP synthase is a key enzyme in cellular energy interconversion. During ATP synthesis, this large protein complex uses a proton gradient and the associated membrane potential to synthesize ATP. It can also reverse and hydrolyze ATP to generate a proton gradient. The structure of this enzyme in different functional forms is now being rapidly elucidated. The emerging consensus is that the enzyme is constructed as two rotary motors, one in the F_1 part that links catalytic site events with movements of an internal rotor, and the other in the F_0 part, linking proton translocation to movements of this F_0 rotor. Although both motors can work separately, they must be connected together to interconvert energy. Evidence for the function of the rotary motor, from structural, genetic and biophysical studies, is reviewed here, and some uncertainties and remaining mysteries of the enzyme mechanism are also discussed.

ATP production is one of the major chemical reactions in living organisms. It has been estimated that a human uses 40 kg of ATP in normal daily living. Assuming the pool of nucleotides is 100 mmol, each molecule of ADP in the body must be phosphorylated and the product ATP dephosphorylated an average of 1000 times per day. The enzyme primarily responsible for the production of ATP is the F_1F_0-type ATPase (denoted throughout this article as F_1F_0), also called ATP synthase. This large complex has eight different subunits in prokaryotes and 16–18 in mammals, and a molecular weight of 550–650 kDa. It is found in the plasma membrane of bacteria, the chloroplast thylakoid membrane in plants, and the mitochondrial inner membrane in plants and animals. Interestingly, there are recent reports of an F_1F_0 ATP synthase in the plasma membrane of human endothelial cells; in this case, the enzyme appears to act as the angiostatin receptor [1].

Figure 1 summarizes our current knowledge of the structure of F_1F_0 from *Escherichia coli*. The protein is bipartite as implied by the nomenclature. There is an F_1 part of α_3, β_3, γ, δ and ε subunits, and an F_0 part, comprising *a*, *b* and *c* subunits in the stoichiometry 1:2:10–14. F_1 and F_0 are linked together by two stalks, a central one containing the γ and ε subunits, and a peripheral one involving the δ and *b* subunits. In mammals, the additional subunits are mostly in the stalk region.

The F_1F_0 complex from all sources can be dissociated under mild conditions into the two component parts, with the F_1 still functioning as an ATPase, and the bilayer-intercalated F_0 retaining a proton translocation function, although this is now passive and bidirectional. Only when the two parts are linked is ATP synthesized from energy derived from vectorial proton translocation. Similarly, ATP hydrolysis can only be used to generate a proton motive force in the intact enzyme. This linkage of endergonic and exergonic reactions in F_1F_0 is called coupling. When the interaction between F_1 and F_0 is disrupted so that energy transduction is lost, the system is said to be uncoupled.

Roderick A. Capaldi*
Robert Aggeler
Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA.
*e-mail: rcapaldi@oregon.uoregon.edu

The binding change mechanism, and F_1F_0-mediated ATP synthesis and hydrolysis

The F_1 part contains three catalytic sites, one on each of the β subunits. When ATP is added in substoichiometric amounts so that only one of these catalytic sites is occupied, substrate binding is very tight and ATP hydrolysis occurs very slowly. Addition of an excess of ATP leads to binding in all three catalytic sites, but with a much lower affinity of the substrate at the second and third sites. The K_d for binding ATP at the first site has been measured at <1 nM, whereas that at sites 2 and 3 is ~1 μM and 30 μM, respectively. Upon occupancy of the third site, the rate of overall ATP hydrolysis increases by a factor of 10^4–10^5 [2]. Thus, the F_1 part is effectively a trimeric complex of three α and three β subunits that displays strong negative cooperativity of substrate binding and, at the same time, strong positive cooperativity of enzymatic activity. To explain these unusual properties, Boyer proposed what has become known as the 'binding change' or 'alternating site' hypothesis [3], an iteration of which is shown in Fig. 2. The key feature of this hypothesis is that the three catalytic sites, and therefore the three $\alpha\beta$ subunit pairs containing these sites, are each in a different conformation at any one time. One is open and ready for ATP (or ADP + P_i) binding, while the second and third are partly open and closed, respectively, around bound nucleotide. ATP binding, and the resulting closure of the open site, produces a cooperative conformational change in which the other two sites are altered, so that the closed one becomes partly open and the partly open one becomes fully open. Thus, each site alternates between the three states as ATP hydrolysis or, in the reverse direction, as ATP synthesis, proceeds. Specific details about the number of conformational intermediates that the three $\alpha\beta$ pairs together can adopt, and about the reaction equilibrium of the cleavage or synthesis of ATP in each site in each state, are still being debated [4]. Nevertheless, the general concept is almost universally accepted and has directed much of the research on F_1F_0 over the past two decades [2,5].

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A radical idea: F_1F_0 is a rotary motor

Obvious questions raised by any binding change mechanism are how the conformational changes are propagated between the three catalytic sites in F_1F_0, and how the catalytic site events are coupled to proton translocation. Boyer suggested that both could occur by rotation of one or more of the single copy subunits in F_1 [3,5]. We now know that it is the γ and ε subunits that provide the rotor. The idea that F_1F_0 works as a rotary motor was also developed independently by Cox and colleagues [6], but with a focus on the proton translocation mechanism in the F_0 part. These researchers insightfully proposed that the *c* subunit ring rotates against the *a* and *b* subunits during proton translocation.

The notion that F_1F_0 functions as a rotary motor was met initially with healthy skepticism. The idea is now almost universally accepted (but see Ref. [7]) because of recent experiments that have elegantly combined genetics, X-ray analysis, electron microscopy, video fluorescence microscopy and chemical crosslinking. How the field came to believe in rotation is an interesting story about the application of diverse innovative biochemical and physical approaches to the problem. The findings that have led to our present understanding of the motor mechanism of the ATP synthase are summarized below and issues for further study are highlighted.

Electron microscopy studies: an early indication of γ rotation within the $\alpha_3\beta_3$ ring

In the late 1980s, using cryoelectron microscopy it proved possible to visualize the F_1 part of the *E. coli* complex through the top or bottom, in relation to the picture in Fig. 1. Because of the intrinsic asymmetry of the molecule, and particularly after this asymmetry was accentuated by immunolabeling of the three α subunits with Fab fragments, noisy images could be averaged and details of the subunit arrangement resolved. These analyses showed convincingly that the α and β subunits alternate around a hexagon containing a central mass that could be identified as the γ subunit. When the individual images were classified on the basis of their dominant features, they fell into three classes, with the γ subunit being located at a different αβ pair in each [8].

X-ray structure of F_1: a protein made to rotate

The year 1994 saw the publication of a high-resolution (2.8 Å) structure of a major part of the bovine heart F_1, including $\alpha_3\beta_3$ and part of the γ subunit [9]. This seminal study was an important confirmation of basic tenets of the alternating sites hypothesis. It showed an enzyme in which the three catalytic sites had different conformations: one open, one closed for ongoing bond cleavage, and the third partly open for imminent release of product ADP and P_i. Equally interesting, this structure showed the γ subunit extending up through the hexagon as two long α helices in a coiled coil, making only limited contacts

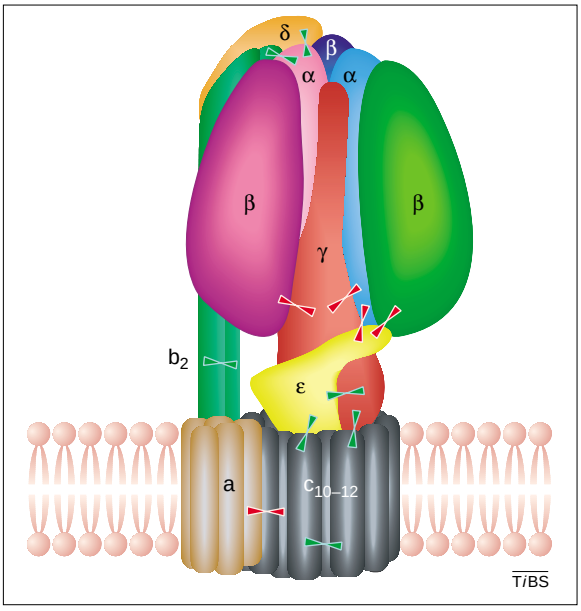

Fig. 1. The arrangement of the subunits in the *Escherichia coli* F_1F_0 ATP synthase complex. One α subunit has been removed from the F_1 part to reveal the γ subunit within the $\alpha_3\beta_3$ domain. There are three αβ subunit pairs, shown here in magenta, blue and green (dark for β and light for α). The $\alpha_3\beta_3$ domain is attached to the *a* subunit in the F_0 part by a peripheral stalk composed of δ and the two copies of the *b* subunit. The *c* subunit ring of F_0 is linked to the γ and ε subunits to form the central rotor. Bows show some of the crosslinks that have been generated to probe the functioning of the enzyme complex. Ones in green have little or no effect on functioning; those in red dramatically inhibit ATP hydrolysis and ATP synthesis.

with the α and β subunits. These contacts included a collar at the top, provided by the N-terminal domains of the α and β subunits, that snuggly fitted the γ subunit, thereby providing a hydrophobic or greasy sleeve – an ideal structure for efficient rotation.

Video fluorescence microscopy: a direct visualization of γ subunit rotation

Once the X-ray structure of F_1 was available to direct experiments, various approaches were employed in quick succession to establish rotation of the γ subunit. It had been shown that crosslinking of γ to the C-terminal domain of β blocked activity [10]. Cross and colleagues extended this work by showing that, if the crosslink was subsequently released and reformed, γ became attached to a different β subunit [11]. Then, with chloroplast F_1, Junge and colleagues established a rotation of γ through at least 280° by fluorescently labeling the C-terminal amino acid of this subunit and then measuring ATP hydrolysis-driven rotation by polarized absorption recovery after photobleaching [12]. The defining experiment (which drew gasps and spontaneous applause at meetings on ATPase) was accomplished by Noji *et al.* [13], who had the idea of attaching a fluorescently labeled actin filament to the γ subunit and watching the movements of this filament by video fluorescence microscopy. This group saw that ATP hydrolysis drove a 360° rotation of the actin filament in three 120° steps in one direction (counterclockwise when viewing the enzyme from below in Fig. 1) with very few

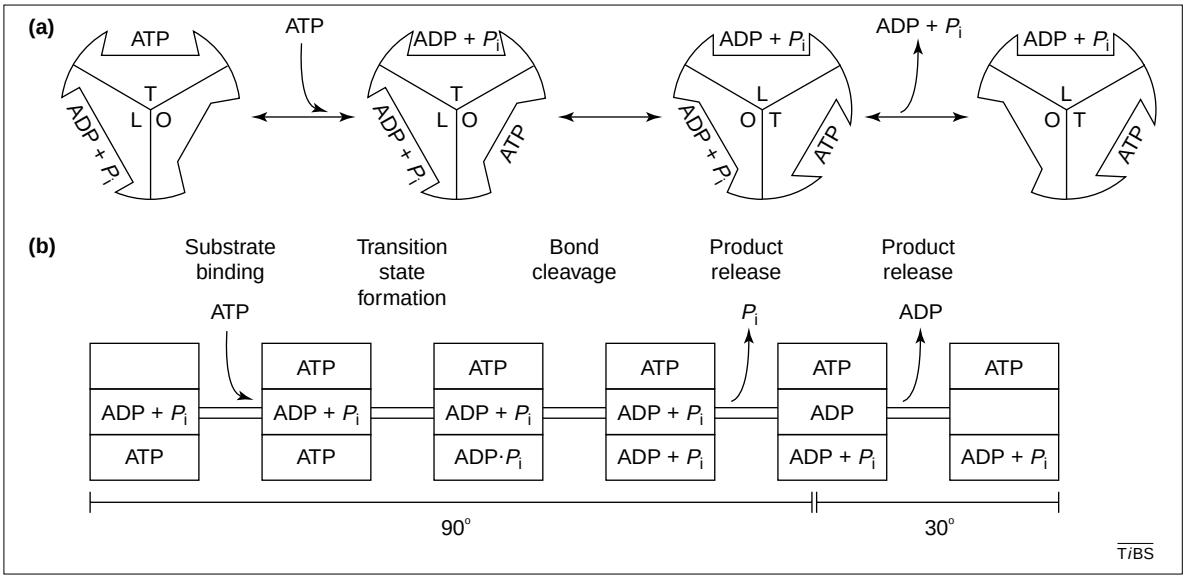

Fig. 2. The alternating site hypothesis and catalytic site linkage to rotation of the γ subunit. (a) One iteration of the alternating site or binding change mechanism taken from Ref. [11]. Each catalytic site cycles through three states: T, L and O. ATP binds to the O (open and empty) site to convert it into a T (tight and ATP-occupied) site. After bond cleavage, the T site is converted into the L (loose and ADP + P_i-occupied) site, from which the products can escape to recover the O state. At any one time, the three catalytic sites are in the O, T and L states, respectively. The concerted switching of states in each of the sites results in the hydrolysis (or synthesis) of one ATP molecule, and a rotation of the γ–ε rotor of 120°. More recently, additional conformations of the T and L states have been assigned on the basis of new crystal structures but with the same 120° overall rotation for each ATP hydrolyzed or synthesized [45]. (b) Substeps in the hydrolysis or synthesis of one ATP molecule based on kinetic and inhibitor studies (reviewed in Ref. [2]) are shown for a three-site mechanism. Thus, ATP binding in the open site (top) leads to transition-state formation and then bond cleavage in a closed site (bottom), followed by P_i release from a partly open site as it opens fully and then releases ADP (middle). Each step in (b) could produce a substep in the rotation of the γ subunit, so that the 90° rotation seen by Yasuda *et al.* [14] is broken up into several steps. This relatively simple scheme has only one site forming the transition state, but it has been argued recently that two of the three catalytic sites can assume the transition-state conformation simultaneously [46].

reversals. (Videos of this dramatic experiment can be seen at www.res.titech.ac.jp/seibutu/main_.html). Because of the viscous drag on the actin filament, the rate of rotation was very slow – only 3% of the enzyme turnover rate. This problem has been largely overcome in recent experiments by tagging the γ subunit with much smaller (40 nm) gold particles. In their latest study, Kinosita, Yoshida and colleagues, observed rotation rates of 134 revolutions per second, which is the rate expected at steady-state ATP hydrolysis under the conditions used (23°C, 2 mM ATP). The researchers were then able to dissect out two substeps in the rotation of γ between two αβ pairs, one of ~90°, which they attribute to ATP binding, and a second of ~30° which, they argue, occurs with product release [14]. These two substeps were separated by a short dwell time.

Crosslinking defines the rotor and the stator

Crosslinking studies have been important for our present pictures of ATP synthase structure and function. The earliest experiments used chemical modifying reagents to induce crosslinking [15], but these were soon superseded by genetic incorporation of pairs of cysteine residues into parts of the complex (for example, see Ref. [10]). Figure 1 shows some of the crosslinks that have been generated between subunits in the ATP synthase complex of *E. coli* and their effects on activity (those shown in red inhibit ATP hydrolysis; those in green have no effect). It is these crosslinking studies that first defined the rotor composition fully by showing that γ, ε and the *c* subunit ring must rotate together: they can be covalently interconnected without loss of ATP hydrolysis-driven proton pumping or ATP synthesis [16,17]. Crosslinking within the $\alpha_3\beta_3$, δ, *a* and *b* subunits was also obtained in some cases without loss of function. For example, the δ subunit can be covalently linked to α without affecting ATPase activity or proton pumping [18,19], as would be expected if the δ and *b* subunits form a stator. This is not to say that the stator is a rigid linkage between $\alpha_3\beta_3$ and the *a* subunit; indeed, there is clear data to show that the flexibility of the stator is important for enzyme function [20]. Fillingame and colleagues conducted extensive crosslinking studies within the *c* subunit ring, and between the *c* subunit ring and both the *a* and *b* subunits. All are consistent with the *c* subunit ring rotating as a unit against the ab_2 part [21]. More recently, an energy dependence of the rotation of the *c* ring relative to *a* has been demonstrated by Cross and colleagues [22].

Rotation of the *c* ring is difficult to demonstrate unequivocally

Direct visualization of the rotation of the *c* subunits against the *a* subunit is necessary to dissect out the kinetics and stepping as a function of enzyme turnover. Stepping here refers to the number of discrete rotational steps of the *c* subunit ring against the *a* subunits for each 120° rotation of the γε subunit pair with respect to the αβ pair in F_1. However, demonstrating rotation of the *c* subunit ring in the

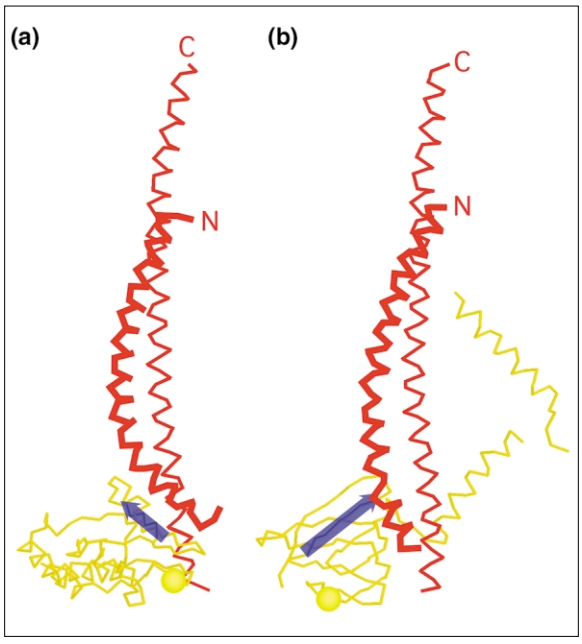

Fig. 3. Rotation involves unwinding of the coiled-coil α-helical part of the γ subunit. The arrangement of γ and ε subunits in the DCCD-inhibited mitochondrial F_1 structure (a) and in the *Escherichia coli* F_1 structure (b) are compared. Only the N-terminal (thicker red line) and C-terminal α helices of the γ subunit are presented. It can be seen that the coiled coil of these two α helices is significantly unwound and rotated in *E. coli* F_1 compared with the mitochondrial structure. This results in a complicated movement of the ε subunit (yellow), including a rotation through 81° and a translation of 23°. The blue arrow shows this rotation of ε by following the orientation of β strand 1 of the β sandwich-like N-terminal domain of this subunit. (This rotation is clearer in the projection through the bottom of the F_1 shown in Fig. 4.) The yellow ball shows the position of His38 (*E. coli* numbering system). There is also an important difference in the arrangement of the C-terminal domain of ε in the two structures. In the structure in (a), the helix–loop–helix C-terminal domain extends outwards and sideways towards the *c* subunit ring (this subunit is called δ in mitochondrial F_1, as in Ref. [30]). In the arrangement in (b), the two α helices of the C terminus of ε are apart and extend upwards for interaction of the C-terminal α-helix with α and β subunits, as first shown by the structure of the isolated γε complex from *E. coli* [47]. This different arrangement has functional significance. In *E. coli* F_1F_0, the ε subunit has dual functions, one in coupling and the other as an inhibitor of ATPase activity [48]. In mitochondrial F_1F_0, an intrinsic inhibitor protein controls ATPase activity [49], and the C terminus of δ (*E. coli* ε) is blocked from reacting with the α and β subunits by its close interaction with a subunit (unfortunately called ε) [30], which is not present in the bacterial enzyme.

intact F_1F_0 complex is proving problematic. Several groups have reported visualizing ATP-driven rotation of the *c* subunit ring when monitored by the fluorescent actin filament method [23–25]. However, this rotation could be artefactual. In the experiments of Sambongi *et al.* [24], the few rotations of the *c* subunit ring that were observed were only marginally sensitive to the inhibitor venturicidin and not at all sensitive to the inhibitor DCCD. (An observed effect of the hydrophobic carbodiimide DCCD is the 'gold standard' for demonstrating coupling, as this reagent reacts with Asp61, a buried carboxyl in the *c* subunit that is absolutely required for proton translocation.) If rotation is observed in a preparation that is insensitive to DCCD, the system is probably uncoupled; for example, by disruption of the interaction between the *c* subunit and the *a* subunit, against which it must rotate to drive proton translocation [25]. In a recent study, Junge *et al.* [26] have observed rotation of the *c* subunit ring in an enzyme preparation in which proton translocation is lost and coupling thereby blocked by mutation of Asp61 to Asn. This is strong evidence that the rotation of the *c* ring observed in the actin filament-type studies is not a valid reflection of the rotation of the γ–ε–*c* ring-rotor in the fully functional enzyme.

More work is needed to set up an experimental system in which to study *c* subunit rotation driven by ATP hydrolysis and, of course, for the ultimate goal of monitoring this rotation when driven by a proton gradient to make ATP. The key problem is that membrane-bound F_1F_0 must be solubilized and purified for actin-filament-type or other single molecule studies. It then requires a lipid milieu to retain functioning. As yet, no detergent has been found that substitutes for lipids fully and, for ATP synthesis studies, a proton gradient is essential. The challenge, which remains to be accomplished, is to use microscopy to examine F_1F_0 molecules individually, widely dispersed in large lipid vesicles.

How are the rotations of γ–ε and the *c* ring coupled to ATP hydrolysis?

Current research is now shifting away from 'does F_1F_0 work as a rotary motor?' to 'how is this accomplished?' A key consideration is the amazing efficiency of the process within the F_1 part of the enzyme. Based on the actin filament rotation studies, the torque generated during rotation is ~40 pN nm^{-1}, which puts the efficiency close to 100% [27]. As pointed out by Oster and Wang [28], efficiency increases if energetic transactions proceed in small steps and, according to thermodynamic principles, the more the better. The implication is that for each 120° turn, there must be several substeps linked to the catalytic site reaction, each conveyed to the γ subunit by a different conformational change in the $\alpha_3\beta_3$ ring. Consideration of the reaction scheme for ATP hydrolysis (Fig. 2b) indicates several possible mechanochemical steps: ATP binding, transition state formation, bond cleavage, P_i release and ADP release. Several or all of these steps can alter the conformation of αβ pairs to drive or facilitate rotation of the γ subunit. As reviewed briefly above, Yasuda *et al.* [14] were able to resolve two substeps – a 90° and a 30° rotation of γ – in their latest experiments. These researchers argue that the 90° rotation is brought on by ATP binding, whereas the 30° rotation occurs with ADP release. There is evidence that the bond cleavage step, $E{\cdot}ATP \Leftrightarrow E{\cdot}ADP + P_i$, affects the arrangement of the γ subunit and, thus, along with ATP binding and ADP and/or P_i release, helps drive the rotation. Thus, when Cys residues introduced at residues 8 or 106 are labeled with a fluorescent dye [29], there is a fluorescence shift on binding ATP to a single catalytic site, which is reversed in part by the bond cleavage reaction at that site before product release. Other

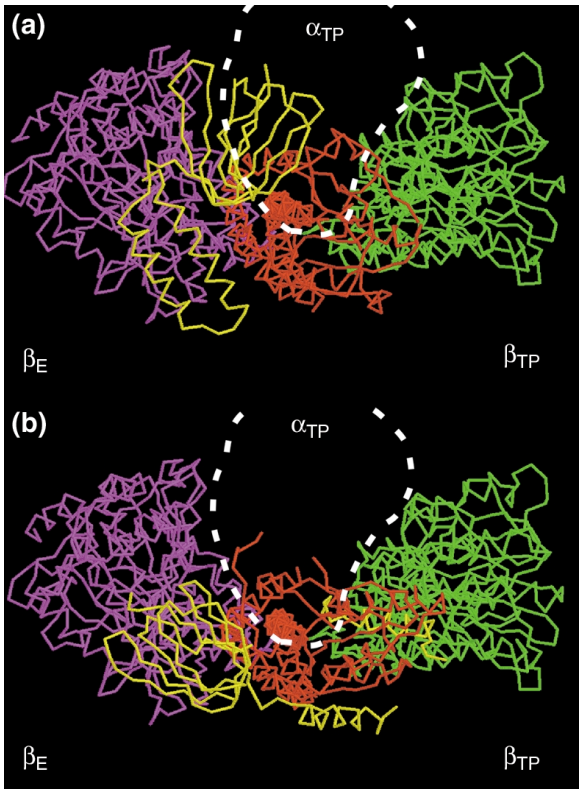

Fig. 4. Rotation of the γε domain visualized in crystallography. The different positions of the ε subunit in DCCD-treated MF_1 and *Escherichia coli* F_1 are clearly visualized when the two structures are examined in the hexagonal projection of the $\alpha_3\beta_3$ domain; in this case, from the bottom relative to F_1 as shown in Fig. 1. The color scheme is the same as in Fig. 1, and nomenclature for the different αβ pairs is as proposed by Abrahams *et al.* [9]. For clarity, the α_{TP} subunit is shown as a profile only (dotted line). β_E contains the open catalytic site; β_{TP} and α_{TP} contain a closed catalytic site. The ε subunit moves from below α_{TP} in the structure of DCCD-inhibited mitochondrial F_1 (a) to below β_E in ECF_1 (b), a rotation of 81°.

substeps of ATP hydrolysis or ATP synthesis might contribute to the rotation of γε. It is well established that P_i binding is the key energy-requiring step for ATP synthesis [2], and we suggest that this step also contributes so that there are at least four conformational states to provide substeps in the overall 120° rotation.

Insights into the conformational changes involved in the movements of the rotor within F_1 are now coming from comparisons of recently reported structures of the enzyme. For example, a comparison of the structure of the DCCD-inhibited form of mitochondrial F_1 [30] with that of the recently described 4.4 Å structure of *E. coli* F_1 [31] reveals significant domain movements of the rotor. Specifically, there is an unwinding of the two large α helices in γ to rotate the ε subunit by ~80° (Fig. 3), along with translocation of ε by ~25 Å from below an α subunit to below a β subunit (Fig. 4). A similar shift in γ and ε is seen when the DCCD-treated mitochondrial F_1 structure is compared with that of a partial complex of the yeast F_1F_0 ($\alpha_3\beta_3\gamma\varepsilon c_{10}$) [32]. Unfortunately, the identity of the nucleotides present in individual catalytic sites is only known for the DCCD-treated MF_1 and not for the ECF_1 nor the yeast F_1F_0 structures. Therefore, it is not yet possible to be precise about which of the catalytic substeps described in Fig. 2 drive the observed rotation.

The best evidence that the different arrangements of γ and ε seen in the X-ray studies are functionally relevant and not caused by crystallization artefacts comes from cryoelectron microscopy studies. When the ε subunit in isolated ECF_1 is labeled with a 14 Å-nanogold particle at a Cys introduced to replace His38, and the preparation rapidly frozen (to –170°C) immediately after adding ATP + Mg^{2+}, the ε subunit is found below a β subunit [33]. However, when turnover is allowed to proceed to generate ADP before the freezing step, the ε is below an α subunit, a translation of ~25 Å and, therefore, of the same dimensions seen in the comparison of the two crystal structures. Nucleotide-dependent movements of the ε subunit from below an α subunit to below a β subunit have also been observed in crosslinking experiments [34].

Mechanism of rotation of the F_0 rotor: movements of the *c* ring against subunit *a*

The least well-defined part of the ATP synthase is F_0. In contrast to F_1, there are no high-resolution structures of F_0 on which to base mechanistic models. At present, models of F_0 structure rely on nuclear magnetic resonance-based structures of the monomer *c* subunit [35], along with both the recent moderate-resolution X-ray data of the partial yeast F_1F_0 complex [32] and atomic force microscopy studies [36–38], all of which support the ring arrangement shown in Fig. 1. Mapping of the putative proton channel at the interface between the *c* and *a* subunits is based on mutagenesis studies that have identified Asp61 of *c* and Arg210 of *a* as crucial amino acids for proton translocation [39,40].

An important contribution to our present understanding of the structure and function of F_0 has been the work of Dimroth and colleagues on the enzyme from the bacterium *Propionigenium modestum* [41]. The F_1F_0 from this bacterium can pump Na^+ as well as protons, facilitating binding studies of the translocating ion while the enzyme is active. Kaim and Dimroth have generated mutants in which Na^+ translocation is abolished while Li^+ or H^+ translocation is retained; subunit *a* has the following changes: K220R, V264E, I278N. They find that Na^+ inhibits ATP hydrolysis in this mutant as a result of entrapment of one cation in the sodium–proton channel [42]. This is strong evidence that the F_0 part acts as a single channel. Current models of proton translocation through F_0 have either a single channel at the *a*–*c* subunit interface or two half-channels, as shown in Fig. 5. In a two-half-channel model [43], each *c* subunit is protonated at Asp61 through one-half-channel as this monomer comes into contact with the *a* subunit. The protonated *c* subunit then rotates 360° before the proton is released through the second half-channel and comes into contact with the *a* subunit again.

Any detailed explanation of mechanism obviously requires a knowledge of the number of *c* subunits in the ring, but this is proving extremely difficult to define unambiguously. Accumulating evidence implies that the stoichiometry of *c* subunits is variable between different species and perhaps even variable in an individual species depending on metabolic conditions [44]. According to X-ray data,

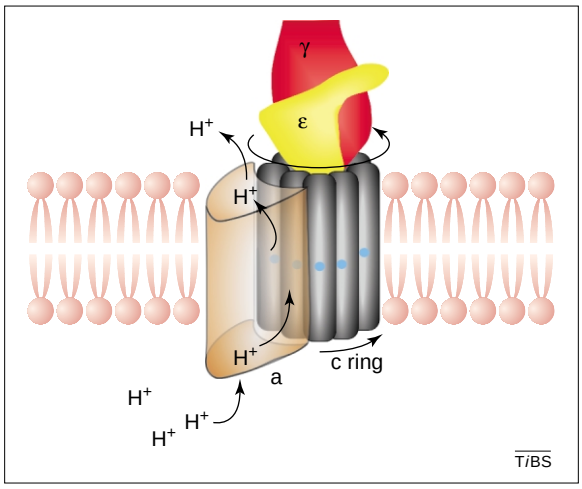

Fig. 5. A two-channel mechanism for proton translocation in the F_0 rotary motor. The ε subunit, *c* subunit ring and *a* subunit are shown, along with part of the γ subunit (same color scheme as in Figs 1 and 3). In this model, proton movements across the membrane (towards the F_1 part) drive rotation of the γε–*c* subunit ring in steps, each representing the movement of one *c* subunit into, and a second *c* subunit out of, an interaction with the *a* subunit.

there are 10 *c* subunits in yeast [32]; a value of 10 has also been obtained recently for *E. coli* by crosslinking and genetic studies [21]. By contrast, atomic force microscopy has generated values of 11 and 14 for *P. modestum* and chloroplast F_0, respectively [38].

How are 10–14 steps of the rotor in F_0 synchronized with 3×120° steps in F_1?

A key issue raised by the odd stoichiometry of subunits in F_1F_0 is how the F_1 and F_0 motors are synchronized for efficient energy transfer between the two. If each *c* subunit translocates one proton, and if it takes the energy for translocation of three or four protons to synthesize one ATP molecule, then three or four small rotations (each through an angle that depends on the number of *c* subunits in the ring) are required to drive a 120° rotation of γε relative to the $\alpha_3\beta_3$ domain. One possibility, as outlined in Fig. 2, is that the movement of each *c* subunit is separately linked to a substep of γε rotation, and hence to a partial reaction in ATP synthesis (or ATP hydrolysis). An alternative to such a stepping mechanism is that the energy of each *c* subunit movement is stored within the protein until it is sufficient to move γε through a 120° rotation with the resulting synthesis of one ATP molecule. This alternative has been called an elastic strain mechanism [26] and it has been suggested that elastic energy could be stored in the stator, which is known to have considerable flexibility.

The recent dissection of partial steps in the 120° rotation of γε by Yasuda *et al.* [14] seems to favor a stepping mechanism. However, it is difficult to accommodate a variable stoichiometry of *c* subunits in such a mechanism. By contrast, a variability of the number of *c* subunits in the ring and, therefore, a variable ratio of protons translocated per ATP synthesized or hydrolyzed, is easily accommodated in an elastic strain type of model.

Conclusions

Our understanding of the functioning of ATP synthase has advanced dramatically in the past decade to the point where the enzyme is now being studied by physicists interested in developing nanomachines for information storage and energy interconversion. However, the mechanism of ATP synthase is far from fully understood, and there are several experimental challenges to be met as outlined in this article. As with many biophysical problems, which is what mechanistic studies of F_1F_0 have largely become, progress is tied to the development of new technologies or the improvement of old ones. Fortunately, advances in methodology for single molecule studies are coming rapidly and there is an increasing number of laboratories ready and able to apply the new techniques to what Boyer has called 'nature's splendid molecular machine'.

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