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Biological nano motor, ATP synthase F_oF_1: from catalysis to $\gamma\varepsilon c_{10-12}$ subunit assembly rotation

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Abstract

Proton translocating ATPase (ATP synthase), a chemiosmotic enzyme, synthesizes ATP from ADP and phosphate coupling with the electrochemical ion gradient across the membrane. This enzyme has been studied extensively by combined genetic, biochemical and biophysical approaches. Such studies revealed a unique mechanism which transforms an electrochemical ion gradient into chemical energy through the rotation of a subunit assembly. Thus, this enzyme can be defined as a nano motor capable of coupling a chemical reaction and ion translocation, or more simply, as a protein complex carrying out rotational catalysis. In this article, we briefly discuss our recent work, emphasizing the rotation of subunit assembly ($\gamma\varepsilon c_{10-12}$) which is formed from peripheral and intrinsic membrane subunits.

1. Catalysis and its structural basis of F_oF_1

The ATP synthase has two major sectors, F_1 and F_o: the membrane peripheral F_1 has three catalytic sites, whereas F_o forms a transmembrane proton pathway. The fundamental subunit structure of this enzyme is well conserved among mitochondria, chloroplasts and bacterial membranes (Fig. 1). F_1 is formed from 5 different subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ [1–3]. The X-ray crystal analysis of the bovine F_1 sector revealed the high resolution structure of the $\alpha_3\beta_3\gamma$ assembly [4]. The three copies of the α and β subunits are located alternately around amino and carboxyl terminal α helices of the γ subunit. Furthermore, an important finding is that the three β subunits are in different states of nucleotide binding: βT, ATP bound; βD, ADP bound; βE, no nucleotide. This apparently asymmetric structure of the β subunits is consistent with the catalytic properties of the enzyme. ATP hydrolysis by F_1 exhibits three different K_m values for ATP, of the order of 10^{-9}, 10^{-6}, and 10^{-4} M, respectively. The rate for a single catalytic site (unisite catalysis) is very low (10^{-3} s^{-1}), but it is stimulated $\sim 10^5$ fold upon binding of ATP to the other two sites [5]. From extensive kinetic studies, Boyer proposed the 'binding change mechanism' for ATP hydrolysis and synthesis [3]. In this mechanism, the product release from one catalytic site is stimulated upon substrate binding to the other sites. The structural and functional basis for the cooperativity is that each β subunit communicates its nucleotide binding state to the other β subunits through α/β interfaces and the centrally located γ subunit. It should be noted that ATP

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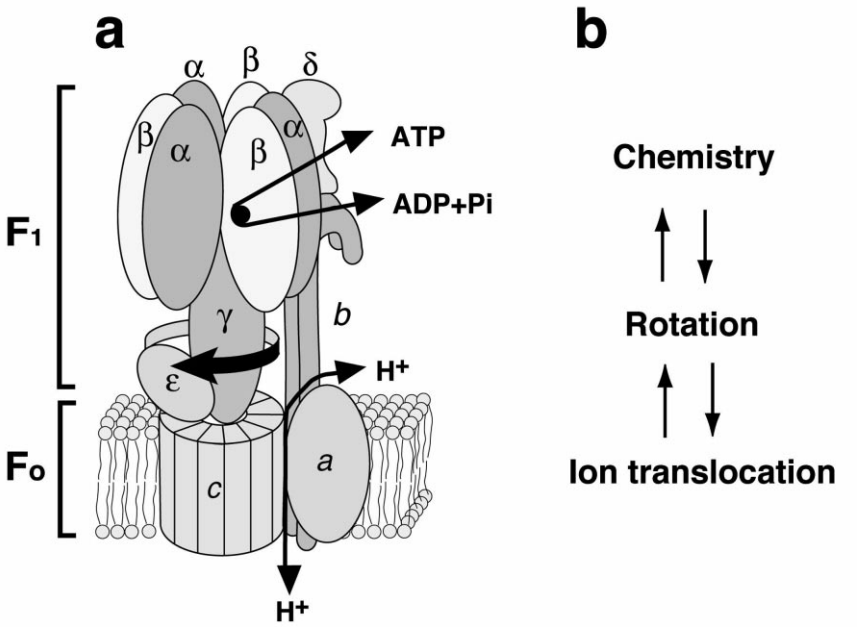

Fig. 1. A concept of ATP synthase F_oF_1. (a) Schematic subunit organization of ATP synthase. Proton translocation and catalysis are shown schematically. (b) Three key steps in ATP synthase energy transformation. The enzyme couples chemistry, mechanical rotation, and ion translocation.

synthesis is essentially dependent on catalytic cooperativity, as the equilibrium constant in unisite catalysis (F_1-ADP ↔ F_1-ADP-Pi) is almost unity [3].

Genetic and biochemical studies revealed that β subunit residues βLys-155 (*Escherichia coli* numbering) [6–9] and βThr-156 [8] in the conserved glycine rich P loop (GX4GKT) together with βGlu-181 [10] and βArg-182 [11] in the GERXXE sequence are catalytic residues. βLys-155 is near the β/γ phosphate moiety of ATP and βGlu-181 can activate a water molecule when then enzyme hydrolyzes ATP [2,3]. The crystal structure of bovine mitochondrial enzyme [4] supports these predictions. Furthermore, βGlu-185, the last residue of GERXXE sequence, and αArg-376 of the α subunit are located at the catalytic site close to the Mg. Mutations in these residues affect ATP synthesis and steady-state ATPase activity, but have essentially no effect on the unisite catalysis of ATP hydrolysis [12–14]. Thus, they are responsible for catalytic cooperativity of the enzyme. Mutations of amino acid residues located at the α/β interface showed similar effects, suggesting that conformation transmission between α and β subunits is required for the catalytic cooperativity [2,14,15].

The F_o sector is formed from three subunits with a stoichiometry of $a_1b_2c_{10-12}$. *c*Asp-61 and *a*Arg-210 of the *c* and *a* subunit, respectively, have been shown to be responsible for proton translocation [16,17]. A ring structure of the 12 *c* subunit oligomer was proposed [18], and suggested by electron microscopy [19,20], atomic force microscopy [21,22], and modeling from an NMR structure of a single *c* subunit having two transmembrane α helices [23]. More recently, the ring structure has been shown by X-ray diffraction of a complex of yeast F_1 and a *c* subunit oligomer: the ten *c* subunits are packed tightly forming two rings of single α helices [24]. Unexpectedly, the X-ray structure shows a ring of 10 *c* subunits, not 12 as expected [24]. The six to seven *c* subunit inter helical loops interact closely with γ and ε subunits of the F_1 sector.

2. Converting chemical energy to γ subunit rotation in F_1 sector

The high resolution structure indicates that the γ subunit interacts with the three β subunits differently [4]. In agreement with the structure, mutations in the γ subunit often inhibit ATP synthesis/hydrolysis, or affect energy coupling, and one of these mutations

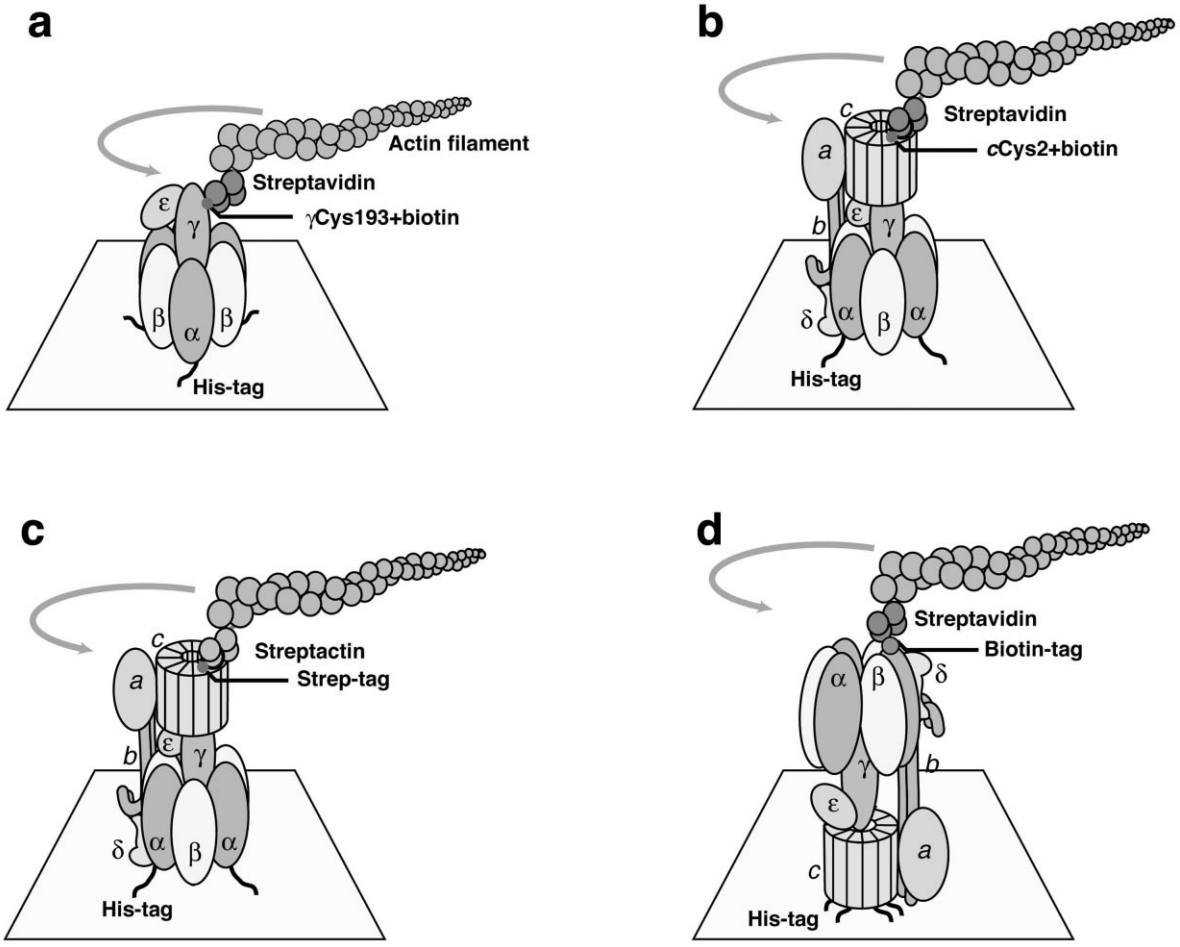

Fig. 2. Observation systems for mechanical rotation in ATP synthase F_oF_1. F_1 (a) or F_oF_1 (b–d) was immobilized through His tag attached to the α subunit (a–c), or to the *c* subunit (d), and a fluorescent actin filament was connected to the γ (a), *c* (b,c), or α (d) subunit for rotation assay: (a) a system for the γ subunit rotation [31]; (b,c) systems for the *c* subunit rotation [45,47]; (d) a system for α subunit rotation.

was suppressed by a second mutation in the β subunit [25]. Furthermore, a mutation in amino terminal region was suppressed by those in the carboxy terminal [26]. Similarly, mutations in the carboxyl terminus were suppressed by those in amino terminus [27]. As those two regions are not interacting directly with each other, the results observed are the long distance suppressions, suggesting a large conformational change of the γ subunit during catalysis.

As the β subunit should go through sequential conformational changes (β_T, β_D, and β_E) during catalysis, the γ subunit should change its relative position sequentially within the $\alpha_3\beta_3$ hexamer [4]. The most likely mechanism for this positional change is the rotation of γ subunit itself in the $\alpha_3\beta_3$ hexamer. The γ subunit rotation has been suggested by elegant biochemical experiments, including chemical cross-linking of the β/γ subunits [28] and polarization recovery after photobleaching of a probe linked to the γ subunit [29]. The rotation during ATP hydrolysis was video-recorded by the rotation of an actin filament connected to the γ subunit of thermophilic *Bacillus* F_1 [30]. In this experiment, F_1 was immobilized on a glass surface through a His tag inserted to the β subunit amino terminus. The torque generated by rotary movement was approximately 40–50 pN nm, the value being comparable to the free energy released from physiological ATP hydrolysis. Therefore, the $\alpha_3\beta_3\gamma$ complex is a very efficient molecular motor converting chemical to mechanical energy.

We could also show that the actin filaments attached to *E. coli* F_1 γ subunit could rotate and generate similar torque to that observed for the thermophilic enzyme (Fig. 2a) [31]. It should be noted that the *E. coli* experimental system has advantage for further studies. As summarized previously [1,2], a collection of various mutants with altered catalysis and energy coupling is available and new mutants can be easily constructed. The mutant enzymes will be powerful tools for dissecting the rotational catalysis.

We have been interested in one class of mutants including γM23K (replacement of γ subunit Met-23 with Lys). The γM23K mutant enzyme can hydrolyze ATP essentially similarly to the wild-type, but could not form a proton electrochemical gradient dependent on ATP hydrolysis [26,27,32]. This uncoupled mutant, however, is still able to rotate the actin filament and produce rotary torque comparable to the wild-type enzyme [31]. This observation indicates that the energy uncoupling is not always due to inefficient torque generation in the $\alpha_3\beta_3\gamma$ complex.

3. Rotation of $\varepsilon\gamma c_{10-12}$ subunit assembly

3.1. Rotation of the c subunit oligomer

The next obvious question is how ATP hydrolysis-dependent γ rotation couples to proton translocation through F_o sector, or proton translocation through F_o drives the γ subunit rotation during ATP synthesis. The F_1 portion is separated from the proton conducting F_o, as shown by electron microscopy of F_oF_1 [33] or the crystal structure of a complex of F_1 and the *c* subunit oligomer [24].

There are two obvious models for the interaction of the γ subunit with F_o sector during ATP synthesis/hydrolysis. One model predicts that the γ subunit interacts with the hydrophilic loop of each *c* subunit one by one during rotation. According to this model, γ slides on the surface of the *c* subunit oligomer which is fixed relative to γ. One of the most convincing pieces of evidence against this model is from chemical cross-linking experiments. Cross-linking between γ and *c* subunit does not affect activities of F_oF_1 [34], indicating that sequential interaction of multiple *c* subunit with the γ subunit is not necessary during ATP synthesis or hydrolysis.

Another model is that the γ and *c* subunit oligomer rotates together as a $\gamma\varepsilon c_{10-12}$ subunit assembly. The results of chemical cross-linking studies support this model: cross-linking between γ and ε, as well as γ and *c*, did not affect ATP hydrolysis [35–39], whereas α–γ, α–ε, β–γ, or β–ε cross-linking resulted in loss of the activity [28,40–43]. The ε subunit is shown to be a part of the rotor when F_1 was subjected to direct observation of rotation [44]. Finally, the rotation of *c* subunit oligomer was demonstrated by the actin filament rotation chemically connected to the *c* subunit(s) of *E. coli* F_oF_1 (Fig. 2b) [45]. In this experiment, His-tag was introduced into the α subunit, a Cys residue was introduced to the amino terminus of the *c* subunit, and all γ subunit Cys residues were replaced by Ala. The modified enzyme was immobilized to a glass surface via Ni^{2+}-NTA-horseradish peroxidase through His-tagged α subunits. The introduced Cys residues in the *c* subunits were used to connect to biotin-maleimide, and fluorescent actin filament was attached to the *c* subunits via streptavidin. Upon addition of ATP, the rotation of the filament was observed under a fluorescent microscope (Fig. 3). The frequency of the rotating filament was slightly lower than when it was directly connected to the γ subunit so as to observe its rotation in F_1 alone. With careful control experiments, we concluded that the filament connected to the *c* subunit rotated. The rotary torque generated by F_oF_1 was calculated to be approximately 40 pN nm, a value similar to that obtained from F_1 γ rotation study [31,45].

The following observation further confirmed that the actin filament connected to the *c* subunit oligomer was rotated: the filament tends to pause frequently in the presence of venturicidin, a specific inhibitor that interacts with the *c* subunit [46]. ATPase activity of F_oF_1 with a *c* subunit (*c*I28T) mutation was less sensitive to this reagent. We have observed that the rotation of the mutant enzyme was virtually unaffected by venturicidin (M. Tanabe, Y. Sambongi, Y. Iko, A. Iwamoto-Kihara, Y. Wada and M. Futai, submitted for publication). DCCD is normally a potent inhibitor of the F_oF_1 ATPase, but we found it to be ineffective at inhibiting either ATPase activity or rotation in the reaction mixture used. This we

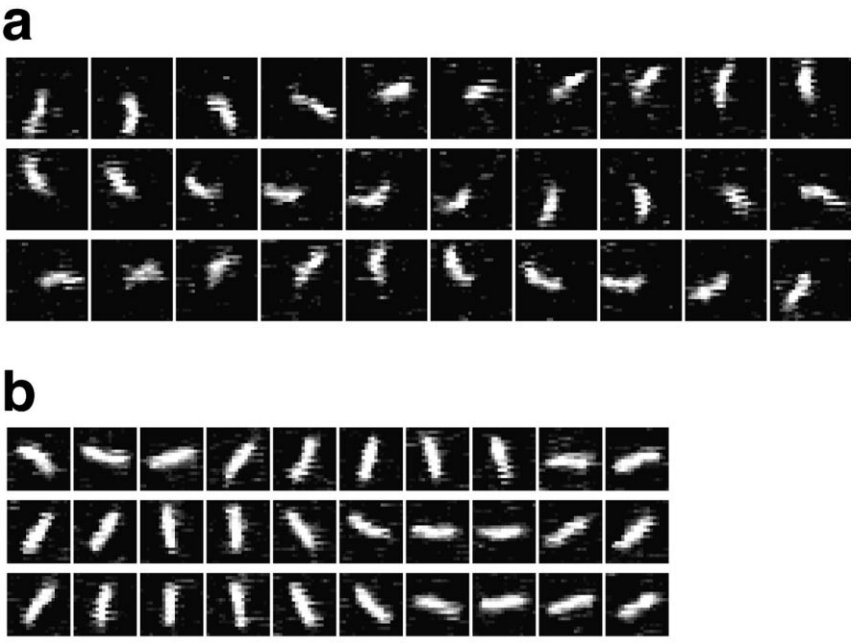

Fig. 3. Rotation of an actin filament connected to the *c* subunit of ATP synthase F_oF_1. Rotation of the actin filament (3.6 μm (a), 3.7 μm (b)) were recorded in the presence of 5 mM MgATP. Experimental system used is described in Fig. 2b.

attribute to the presence of a high concentration of bovine serum albumin, detergent and enzymes for ATP generation.

Most recently, Pänke et al. demonstrated the rotation of the *c* subunit oligomer in a different experimental system [47]. They used a modified version of the *c* subunit in which the carboxyl terminus was tagged with a short peptide ('strep-tag') (Fig. 2c). The strep-tag has a high affinity for streptactin (a modified streptavidin), thus allowing connection of F_o *c* subunits to a biotinylated actin filament. In this approach [47], they could eliminate chemistry including Cys modification with maleimide. Although specificity of chemical modification is high and could be checked by gel electrophoresis followed by blotting with horseradish peroxidase–streptavidin staining, extensive control experiments were necessary to prove the rotation. Therefore, the system entirely relying on molecular biological modifications is advantageous for further studies on the mechanism and energetics of the rotation.

Rotation of the *c* subunit oligomer has been demonstrated by two different experimental approaches. It should be noted that the torque generated by the *c* subunit oligomer and γ subunit was very similar (40–50 pN nm), indicating that the rotary movement of the γ subunit can be transmitted to F_o domain without loss of energy [45,47]. These results suggest that $\varepsilon\gamma c_{10-12}$ assembly rotates and other subunits form a stator. The X-ray structure of the yeast F_1 and the *c* subunit complex suggests that γ and *c* are tightly bound during rotation [24].

3.2. Further studies on a nano motor

It is logical to assume that the stator and rotor could be interchangeable because no part of F_oF_1 is immobilized in the membrane. Thus, stator subunits of F_1 such as α and β should rotate when the rotor $\varepsilon\gamma c_{10-12}$ assembly is immobilized. We examined this question by fixing the *c* subunit on a glass surface and connecting an actin filament to the α subunit (Y. Sambongi, Y. Iko, M. Tanabe, A. Iwamoto-Kihara, L.P. Nga, I. Ueda, Y. Wada and M. Futai, submitted for publication). With this experimental setup, rotation of the filament would be expected only if the $\varepsilon\gamma c_{10-12}$ assembly formed a mechanical unit. The α and *c* subunits were connected with biotin tag (transcarboxylase biotin binding domain) and His-tags, respectively, by genetic engineering; thus no chemistry was required to achieve specificity of the attachment of actin filament (Fig. 2d). The actin

filament, attached to the α subunit, could rotate upon ATP hydrolysis and generate torque of ~40 pN nm. This observation added another line of evidence indicating that F_oF_1 is a rotating enzyme and that rotation of the γ and *c* subunit assembly is an essential feature of the energy coupling between proton transport and ATP hydrolysis/synthesis.

As discussed in this article, ATP synthase is a nano motor which interconverts chemical, osmotic and mechanical energy. One of the most interesting unanswered questions is how γ and *c* oligomer rotation can release ATP from the catalytic site in F_1 and reversibly transport protons through F_o sector. Models with successive interaction between *c*Asp-61 and *a*Arg-210 have been proposed [48,49]. Detailed studies on the rotations of uncoupled mutant enzymes will certainly contribute to the understanding of the molecular mechanism. We are also not far away from showing rotation coupled with the ATP synthesis when electrochemical ion gradient is applied. In this regard, the experimental system (Y. Sambongi, Y. Iko, M. Tanabe, A. Iwamoto-Kihara, L.P. Nga, I. Ueda, Y. Wada and M. Futai, submitted for publication) with the immobilized *c* subunit oligomer (Fig. 2d) is a valuable system to carry out such experiments. The F_o sector of the system can easily be incorporated into a phospholipid bilayer for biophysical studies, but this approach would be more difficult if the F_1 sector were to be immobilized on a glass surface (Fig. 2b,c).

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