**Rotation and structure of \(F_oF_1\)-ATP synthase**

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\(F_oF_1\)-ATP synthase is one of the most ubiquitous enzymes; it is found widely in the biological world, including the plasma membrane of bacteria, inner membrane of mitochondria and thylakoid membrane of chloroplasts. However, this enzyme has a unique mechanism of action: it is composed of two mechanical rotary motors, each driven by ATP hydrolysis or proton flux down the membrane potential of protons. The two molecular motors interconvert the chemical energy of ATP hydrolysis and proton electrochemical potential via the mechanical rotation of the rotary shaft. This unique energy transmission mechanism is not found in other biological systems. Although there are other similar man-made systems like hydroelectric generators, \(F_oF_1\)-ATP synthase operates on the nanometre scale and works with extremely high efficiency. Therefore, this enzyme has attracted significant attention in a wide variety of fields from bioenergetics and biophysics to chemistry, physics and nanoscience. This review summarizes the latest findings about the two motors of \(F_oF_1\)-ATP synthase as well as a brief historical background.

**Keywords:** ATP hydrolysis/\(F_oF_1\)-ATP synthase/high reversibility/rotary motor/stepping rotation.

**Abbreviations:** ADP, adenosine diphosphate; AMP-PNP, adenosine-5’-(β,γ-imino)-triphosphate; ATP, Adenosine-5’-triphosphate; ATP\(\gamma\)S, adenosine 5’-(γ-thio)triphosphate; P\(i\), inorganic phosphate.

\(F_oF_1\)-ATP synthase

Adenosine-5’-triphosphate (ATP) is the ubiquitous energy currency of the cell. The human body contains about 50 g of ATP that is sustained by strict dynamic equilibrium between hydrolysis and synthesis. The total ATP produced under basal metabolism in humans amounts to 50–75 kg per day, and the same amount of ATP is consumed for the large variety of energy-requiring reactions such as muscle contraction, synthesis of biomolecules and mass transfer across biomembranes. Under aerobic conditions, the major ATP synthesis pathway is oxidative phosphorylation of which the terminal reaction is catalysed by \(F_oF_1\)-ATP synthase. This enzyme is found widely in the biological world, including in thylakoid membranes, the mitochondrial inner membrane and the plasma membrane of bacteria. This enzyme catalyses ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate (\(P_i\)) by using the electrochemical potential of protons (or sodium ions in some bacteria) across the membrane, i.e. it converts the electrochemical potential into its chemical form. This enzyme also functions in the reverse direction when the electrochemical potential becomes insufficient: it catalyses proton pumping to form an electrochemical potential to hydrolyse ATP into ADP and \(P_i\). \(F_oF_1\)-ATP synthase is a supercomplex enzyme with a molecular weight of >500 kDa and consists of two rotary motors. One is \(F_1\) (~380 kDa), which is the water-soluble part of ATP synthase. When isolated from the membrane portion, it acts as an ATP-driven motor: it rotates its inner subunit to hydrolyse ATP and is therefore termed \(F_1\)-ATPase. The other rotary motor of ATP synthase is \(F_o\) (~120 kDa), which is embedded in the membrane and generates rotary torque upon proton translocation that is driven by proton electrochemical potential (Fig. 1). Bacterial \(F_1\) is composed of \(\alpha\beta\gamma\delta\varepsilon\) subunits. The three \(\alpha\) and \(\beta\) subunits form the hexameric stator ring in which the \(\alpha\) and \(\beta\) subunits are alternately arranged. The rotor shaft is the \(\gamma\) subunit, which is accommodated in the central cavity of the \(\alpha\beta\) ring. The \(\varepsilon\) subunit bonds onto the protruding part of the \(\gamma\) subunit and provides a connection between the rotor parts of \(F_1\) and \(F_o\). The \(\gamma\) subunit acts as the endogenous inhibitor of \(F_1\) (2–4), by transforming the conformational state from the closed form to extended form that blocks the \(\gamma\) rotation due to steric hindrance (5–8). This inhibitory function is thought to be physiologically important to avoid ATP consumption (9). The \(\delta\) subunit acts as a connector between \(F_1\) and \(F_o\) that connects the stator parts. Thus, the minimum complex of \(F_1\) as a motor is the \(\alpha\beta\gamma\delta\varepsilon\) subcomplex. Catalytic reaction centres for ATP hydrolysis/synthesis reside at the three \(\alpha\beta\) interfaces, which are on the antipodal side of the \(\beta\) subunit as indicated with red circles in Fig. 2A. The non-catalytic ATP-binding sites reside on the other \(\alpha\beta\) interfaces. While the catalytic site is formed mainly with amino-acid residues from the \(\beta\) subunit, the non-catalytic sites are primarily within the \(\alpha\) subunit. Upon ATP hydrolysis on the catalytic sites, \(F_1\) rotates the \(\gamma\) subunit in the antipodal direction viewed from the \(F_1\) side. \(F_o\) part consists of \(\alpha\beta\gamma\delta\varepsilon\) subunits. The number of \(c\) subunits varies among species. For example, the copy number of the \(c\) subunit is eight in bovine mitochondria (10), 10 in yeast (11), Escherichia coli (12) and thermophilic Bacillus PS3 (13), 11 in Ilyobacter tartaricus (14, 15),
Propionigenium modestum (16) and Clostridium paradoxum (17), 13 in thermoalkaliphilic Bacillus TA2.TA1 (18) and Bacillus pseudofirmus OF4 (19), 14 in spinach chloroplast (20) and 15 in Spirulina platensis (21). The c subunits form a ring complex by aligning in a circle. It is widely thought that the c-ring and the α subunit form a proton pathway (for details, see the ‘Proton translocation pathway of F₀’ section). With the downhill proton flow through the proton channel, the c-ring rotates against the αβ subunits in the opposite direction of the γ-subunit of the F₁ motor (22). Thus, in the F₀F₁ complex, F₀ and F₁ push each other in the opposite direction. Under physiological condition where the electrochemical potential of the protons is large enough to surpass the free energy of ATP hydrolysis, F₀, forcibly rotates the γ-subunit in the clockwise direction and then F₁ catalyses the reverse reaction, i.e. ATP synthesis which is the principle physiological function of ATP synthase. In contrast, when the electrochemical potential is small or decreases, F₁ forces F₀ to rotate the c-ring in the reverse direction to pump protons against the electrochemical potential.

**Binding change mechanism and structure of F₁-ATPase**

The three catalytic sites on the β-subunits work cooperatively during catalysis. The classic working model for F₁ is the ‘binding-change mechanism’ proposed by Paul Boyer (23). The early stage of this model postulated alternating transition between two chemical states, assuming two catalytic sites residing on F₁. It was later revised to propose the cyclic transition of the catalytic sites among three catalytic sites based on the biochemical and electron microscopic experiments that revealed that F₁ has the three catalytic sites (24–26). One important feature of this model is that the affinity for nucleotide at each catalytic site is different from each other at any given time, and the status of the three β-subunits cooperatively change in one direction accompanying γ rotation. This hypothesis is strongly supported by X-ray crystallographic studies performed by Walker’s group (27). The first resolved crystal structure of F₁ (27) revealed many essential structural features of F₁ at atomic resolution. Importantly, the catalytic β-subunits differ from each other in conformation and catalytic state; one binds to an ATP analogue, adenosine-5′-(β,γ-imino)-triphosphate (AMP-PNP), the second binds to ADP and the third site is empty (Fig. 2A). Therefore, these sites are termed βTP, βDP and βEmpty, respectively. While βTP and βDP have a closed conformation trapping bound nucleotides on the catalytic sites, βEmpty has an open conformation
swinging the C-terminal domain away from the binding site to open the cleft of the catalytic site (Fig. 2B). These features are consistent with the binding-change mechanism. Another important feature found in the crystal structure is that while the N-terminal domains of the α and β-subunits form a symmetrical smooth cavity as the bearing for γ rotation at the bottom of the α2β3-ring, the C-terminal domains of the β-subunit show distinct asymmetric interactions with the γ-subunit. Therefore, the most feasible inference is that the open-to-closed transition of the β-subunits upon ATP binding pushes the sequential conformational change among β-subunits leads the unidirectional γ rotation, which was recently visualized in simultaneous imaging of the conformational change of the β-subunit and the γ rotation (28).

**Verification of F1 rotation by single-molecule observation**

Since the publication of the crystal structure, many studies have attempted to demonstrate the rotation of F1. Crosslink exchange experiment between the β- and γ-subunits of F1 derived from E. coli (29) and the polarized absorption relaxation of F1 from spinach chloroplasts (30) have proven the rotational motion of the γ-subunit during catalysis. Unidirectional rotation of the γ-subunit upon ATP hydrolysis was proved with the direct observation of F1 rotation from thermophilic Bacillus PS3 (TF1) under the microscope (31). In order to suppress rotary Brownian motion study, F1 was immobilized on a glass surface modified with Ni-nitrotetrazic acid (NTA) thorough the interaction between Ni2+ and the His-tag, which was introduced into the N-terminus of the β-subunit. In addition, a fluorescently labelled actin filament with length of 0.6—4 μm and diameter of 10 nm was attached to the γ-subunit as the rotation marker to magnify the subtle motion of the γ-subunit of which the radius is only 1 nm, which is much smaller than the spatial resolution (∼200 nm) of a conventional microscope (Fig. 3A). Note that in recent studies, other types of probes such as polystyrene beads, gold colloidal beads, gold nanorods, and magnetic beads are frequently used instead of actin filaments because the imaging of fluorescently labelled actin filaments suffers from photobleaching. The rotational direction is always anticlockwise when viewed from the F0 side and, importantly, it was consistent with the expected rotary direction from the crystal structure. Assuming the β-subunit undergoes the conformational transition from PEmpty, PTP, and PDP, each catalytic state propagates in the anticlockwise direction, accompanying the anticlockwise γ rotation. The rotational velocity was far slower than the expected rate from bulk ATPase measurements because of the large hydrodynamic friction exerted on the rotating actin filament. However, this allows us to estimate the torque generated by individual F1 molecules from the hydrodynamic friction that should be in equilibrium with F1’s torque. The torque was determined to be around 40 pN nm. Although this is a rough estimation without consideration of the viscosity increment in the immediate vicinity of surface, the value was recently confirmed to be valid using more precise torque measurements based on fluctuation theorem, which estimates the entropy generation upon the rotation with out assuming the friction coefficient (32). Taking into account that the step size is 120°, each coupled with single ATP hydrolysis turnover as below, F1 works with 80 pN nm, which corresponds to the free energy released from hydrolysis of a single ATP molecule under physiological conditions, suggesting high 100% energy conversion efficiency of F1.

**Stepping rotation of F1**

Many attempts have been made to resolve rotary motion into discrete steps to clarify how the rotation is coupled with each elementary catalytic step of ATP hydrolysis: ATP-binding, hydrolysis and product release. The stepping rotation was first observed in the rotation assay with actin filaments under ATP-limiting conditions, where the ATP-binding process determines the net turnover rate of ATP hydrolysis and rotation.
When [ATP] is well below the Michaelis–Menten constant ($K_M$) of the rotation ($\sim$1 μM), $F_1$ showed discrete 120° steps that were intervened with pauses, consistent with the pseudo 3-fold symmetry of the α3β3-ring (Fig. 3B). The mean dwell time of the pause before the steps was inversely proportional to [ATP], suggesting that each step is triggered by a single event of ATP binding. A histogram of the dwell time showed an exponential decay with the time constant in consistent with the observed mean dwell time, implying that the single event triggers the 120° step (33). The coupling ratio of a single 120° step per ATP was directly confirmed in a later study (34). However, the stepping rotation was not detected at ATP-saturating conditions owing to damping by high viscous friction against actin filaments. Therefore, a very small probe was employed to detect the intrinsic stepping motion of $F_1$. A single gold colloid (40 nm) was attached to the γ-subunit so that viscous friction was negligible, and the maximum rotational velocity reached and exceeded the expected rate from bulk ATPase (35). The discrepancy from bulk ATPase is attributed to some fraction of $F_1$ being in an inactive state, the so-called ADP-inhibited form (36), in the ensemble measurement. In this rotation assay, the 120° step rotation was observed even under ATP-saturating conditions. Near the $K_M$, where time constants for the ATP-binding step and other catalytic steps are comparable, the rotation showed two substeps of which angular displacement were resolved into 90° and 30° (35). In a following experiment, in order to facilitate the analysis of the catalytic dwell, a mutation was introduced at the catalytic site, βE190D (thermophilic Bacillus PS3) that significantly slows the rate constant of hydrolysis step (37). Around $K_M$, the mutant $F_1$ shows six pauses composed of 0° and 80° dwelling positions during rotation, revising the substep sizes to be 80° and 40° (Fig. 3C). Kinetic analyses of the dwell time at 0° and 80° dwelling positions revealed that these substeps are triggered by ATP binding and two consecutive reactions with time constants around 1 ms, respectively. Recent studies have revised the two time constants at 80° dwelling position to be 1.3 ms and $\sim$0.1–0.3 ms (38, 39). One of the two reactions at 80° dwelling position was revealed to be the hydrolysis step in the experiment that employed the aforementioned mutant $F_1$ with slow hydrolysis rate and a slowly hydrolysing ATP analogue adenosine 5’-[(γ-thio)triphosphate (ATPγS) (37). The angular dwelling positions at 0° and 80° are, therefore, termed the binding angle and catalytic angle (Fig. 3C and D), respectively. The angular positions of product release were investigated by adding an excess of ADP or P$_i$ in the solution (38, 40). In the presence of ADP, the rotation was slowed because of lengthening of the dwell time at the binding angle, suggesting that the ADP-releasing angle is at a binding angle. Simultaneous imaging of fluorescently labelled nucleotide with the γ rotation also verified this point: fluorescently labelled ATP is released presumably as ADP after the γ-subunit rotates 240° or more from the angle where the nucleotide is bound to $F_1$. In contrast, in the presence of P$_i$, $F_1$ showed longer pauses at the catalytic angle. Thus, it is thought that the release of ADP and P$_i$ occurs at the binding and catalytic angles, respectively. Another intermediate of $F_1$ at the binding angle was unexpectedly found in the rotation assay at low temperature, $\sim$4°C (40). This reaction showed an extremely high $Q_{10}$ factor of 19, so this reaction is termed the temperature-sensitive reaction (TS). A direct correlation between TS and the ATP-binding or ADP-release step was not found although TS takes place at the binding. Considering the high $Q_{10}$ factor, TS reaction might be some conformational rearrangement before or after ATP binding (41).

**Reaction scheme of $F_1$-ATPase**

As mentioned above, all of the elementary reaction steps were identified to occur at the binding angle or catalytic angle. However, because there are three positions for binding and catalytic angles, it is required for the establishment of the reaction scheme of $F_1$ to determine at which angle each reaction occurs. ADP release was shown to occur at 240°–320°, but most likely at 240°. The angle for hydrolysis was determined using a hybrid $F_1$ carrying a single copy of the aforementioned mutant β-subunit, βE190D (42). This hybrid allows us to identify the hydrolysis angle because the incorporated mutant β-subunit shows distinctly long pauses at two positions. One is at the ATP-binding angle of the mutant β-subunit (0°), and the other one is at +200° from the binding angle. Thus, the hydrolysis angle was determined to be 200°. Note that the pause at 0° is due to the TS intermediate state (41), although it was attributed to ATP waiting dwell in the original report (43). The TS dwell could be confounded as the 320° pause presumably due to experimental error. The timing of P$_i$ release has recently been determined to be at 320° in another type of experiment (39) where $F_1$ was stalled with magnetic tweezers during hydrolysis dwell that was lengthened by βE190D and/or ATPγS. On the basis of the observation that bound ATP or ATPγS undergoes hydrolysis and synthesis in a reversible manner, it was shown that P$_i$ (or thiophosphate) is not released immediately after hydrolysis at 200°. Because the P$_i$ release has to be after hydrolysis and at a catalytic angle, it was concluded that P$_i$ release occurs at 320°. Thus, the present reaction scheme of rotation and catalysis is as follows: ATP binding at 0°, hydrolysis at 200°, ADP release 240° and P$_i$ release at 320° (Fig. 4).

**Correlation of reaction scheme with crystal structure**

While the single-molecule rotation assay revealed that $F_1$ has two stable conformations in pausing at the binding or catalytic angle, current crystal structures show essentially a single conformation. Correlation with the crystal structure remained obscure, although the interpretation of the crystal structure is crucial, especially for theoretical studies. Therefore, attempts have been made to determine whether the crystal structure represents the binding dwell or catalytic dwell.
Fig. 4 Mechnochemical coupling scheme of F₁. Each circle represents the chemical state of the catalytic sites on the β-subunit. The red arrow represents the angular position of the γ-subunit. O, C and C' indicate the open, half-closed and closed forms, respectively. The green catalytic site retains the bound nucleoside as ATP until the γ-subunit rotates 200° from the binding angle (0°). At 200°, the catalytic site hydrolyses ATP into ADP and Pi, each of which is released at 240° and 320°, respectively. The conformation of the β-subunit changes from open to closed upon ATP binding and remains in the closed form until the γ-subunit rotates 240°. At 240°, this β-subunit moves to the half-closed form, and then it returns to the open form with accompanying rotation of the γ-subunit.

ATP synthesis upon reverse rotation of F₁

Although the essential properties and basic mechanochemical coupling scheme of F₁ as an ATP-driven motor have been established, the physiological role of F₉F₁-ATP synthase, that is ATP synthesis, has not been sufficiently studied in single-molecule experiments. If ATP synthesis is a simple reverse reaction of hydrolysis, forcibly reversing rotation of F₁ should lead to efficient ATP synthesis. Two lines of single-molecule experiments have been carried out to investigate this hypothesis. In the first experiment (46), a large number of F₁ molecules were enclosed in an observation chamber and forcibly rotated in the reverse direction with a magnetic bead tweezer system. The synthesized ATP was detected as bioluminescence using the luciferin–luciferase system. Although ATP synthesis upon reverse rotation was clearly demonstrated, the uncertainty of the number of active F₁ molecules in the chamber did not allow a quantitative estimation of the mechanochemical coupling ratio. Therefore, the following experiment focused on a single active F₁ molecule to determine the coupling ratio (34). The technical issue to be addressed was detection of a very small number of ATP molecules generated from a single F₁ molecule. Even if we assume that F₁ synthesizes three ATP molecules per one revolution at 10 Hz for 1 min, the total number of ATP molecule is only 1,800 molecules (3.0 × 10⁻²¹ mol), which is far below the detection limit of the luciferase assay. To address this issue, a microscopic reaction chamber system was developed using a microfabrication technique, which has identically shaped reaction chambers, each of which is a few microns in scale and has a volume of 6 fL (47). Because the extremely small reaction volume resulted in high concentration, it was possible to detect a small amount of reaction product yielded from a single enzyme molecule. A single F₁ molecule was encapsulated in the microchamber to accumulate synthesized ATP molecules (Fig. 5A and B). After forcible reverse rotation with magnetic tweezers, F₁ was released from the tweezers. Because the rotational rate of ATP-driven rotation is proportional to [ATP] under the experimental conditions, one can measure the increment of [ATP] as that of the ATP-driven rotation rate. It was found that while the αβγε subcomplex showed very weak ATP synthesis activity, the αβγεζ subcomplex had highly efficient ATP synthesis, up to ~80% (2.3 ATP molecules per turn) (Fig. 5C). It is likely that the ε subunit stabilizes the protruding portion of the γ-subunit, as seen in the crystal structure, to transmit the applied torque to γ. This result implies that the efficiency of the mechanochemical coupling in ATP synthesis is also high in the whole F₉F₁ complex. High reversibility of mechanochemical coupling is a remarkable feature of the ATP synthase that distinguishes it from other molecular motors; other motor proteins such as kinesin and myosin do not synthesize ATP when the movements are reversed by external force.

Structure of F₉

Bacterial F₉ has the common and simple subunit stoichiometry of abε₁₀₋₁₅, while mitochondrial F₉ has additional subunits: d, e, f, OSCP, F6 and A6L (48). We hereafter focus on the minimum subcomplex of F₉, abε₁₀₋₁₅. The structure of the ε subunit was first resolved in a monomer state by NMR (49). The ε...
subunit has a hairpin structure that is composed of two α-helices and a connecting loop. Crystal structures of the F$_{1}$--c-ring structure (10, 11) and isolated c-ring (14–17, 19–21) revealed that the c subunits form a ring complex by assembling in a circle with the C-terminus pointing outward and the connecting loop towards the F$_{1}$ side (cytoplasmic side in bacteria) (Fig. 6). The b subunit has an N-terminal transmembrane domain. The b subunit forms a homodimer (50) which functions as the peripheral stalk to hold the stator parts of F$_{1}$ and F$_{0}$ to avoid slippage. It was shown that the b subunit has robustness against extensive deletion and insertion at the cytoplasmic helix (51, 52), suggesting that the b$_{2}$ dimer also acts as the elastic connector for smooth torque transmission. The structure of the a subunit remains unclear. It is thought that this subunit has five transmembrane helices (53–55). Because the cavity of the c-ring is too small to accommodate the a subunit and/or the b$_{2}$ dimer, each of which has five or two helices, it is reasonable to assume that the ab$_{2}$ complex resides outside of the c-ring (56, 57).

### Fig. 5 ATP synthesis by reversing F$_{1}$

(A) Schematic drawing of experimental setup. (B) Experimental procedure of ATP synthesis. Active single F$_{1}$ is enclosed in a femtolitre chamber (left). A magnetic bead attached to the γ-subunit is forcibly rotated by magnetic tweezers (centre). Newly synthesized ATP is accumulated in the chamber. The number of synthesized ATP molecules is determined by counting the number of ATP molecules that have been transferred from the a-subunit into the Fig. 6 (right). Each trace is derived from individual F$_{1}$ molecules. Dotted lines indicate slopes of the coupling ratio of 0% (0 ATP/turn), 50% (1.5 ATP/turn) and 100% (3 ATP/turn).

### Fig. 6 Structure of F$_{0}$

The c$_{10}$-ring is from the crystal structure of Na$^{+}$-transport F$_{0}$ from Ilyobacter tartaricus (PDB code: 1YCE). The blue spheres at the middle of the c$_{10}$-ring represent bound Na$^{+}$ ions. Schematic image of stator ab$_{2}$ complex (thin orange and blue) is constructed based on the two-channel model. One half channel is exposed to the periplasmic side and the other to cytoplasmic side. Rotation of the c-ring accompanies the proton-transfer between the a and c subunits. Two c subunit monomers at the interface of the a subunit are shown in red and green, respectively. The shortcut transfer of protons between the c subunits connected to the half channels are blocked with the positive charge of the conserved Arg residue of the a subunit (depicted as the plus symbol).

### Proton translocation pathway of F$_{0}$: the 2-channel model

The mechanical rotation of the c-ring by F$_{0}$ is driven by proton flow through F$_{0}$. Although the structural basis of the proton translocation pathway is unknown, extensive biochemical work on F$_{0}$ subunits has identified several charged residues in the transmembrane helices of the a and c subunits that would be directly involved in proton translocation. Among them, Asp or Glu of the c subunit and Arg of the a subunit, which correspond to cAsp61 and aArg210 of E. coli F$_{0}$, are highly conserved among species and thought to have crucial roles in proton translocation. The crystal structure of the c-ring showed that the Asp residue (Glu in *I. tartaricus F*$_{0}$) of the c subunit resides at the middle of the C-terminal helix. The recent structure of the c$_{10}$-ring from *I. tartaricus F*$_{0}$, which is a Na$^{+}$-transporting F$_{0}$, revealed that the Glu residues are occupied with Na$^{+}$ ions (Fig. 6). Thus, it is well established that this conserved carboxyl residue is one of the proton-binding sites. However, other charged residues are not found in the c subunit in the vicinity of the carboxyl residue, suggesting that the a subunit has proton translocation pathways. The most widely accepted model on proton translocation in F$_{0}$ is the so-called two-channel model, which assumes that the a subunit possesses two proton pathways each of which spans half of the membrane, but towards different sides;
the channels connect the proton-binding site of the c subunit with the periplasmic or cytoplasmic space (58–60) (Fig. 6). Notice each channel has contact with a different c subunit, which are adjacent to each other. In other words, the a subunit interacts with two c subunits, each contacting via a different half channel. The proposed mechanism of proton transfer in ATP synthesis mode is as follows (60–62): a proton enters the half channel exposed to the periplasmic side (or intermembrane space of mitochondria) and is then transferred to the carboxyl residue of the c subunit. This protonation neutralizes the negative charge of the residue, allowing the c subunit to rotate apart from the a subunit towards the surrounding lipid layer. At the same time, the neighbouring c subunit at the anticlockwise side returns from the lipid layer to form contacts with the other half channel, which has a hydrophilic environment to promote deprotonation of the carboxyl residue. The released proton then enters into the cytoplasmic space. The role of the conserved Arg in the a subunit is likely to block the futile rotation of the c subunit without deprotonation by attracting only the deprotonated c subunit with its positive charge (62, 63). In the ATP-driven proton-pumping mode, the sequence of events is reversed.

Rotation of c-ring in Fo

After the direct observation of F1, the verification of the c-ring rotation against the ab2 complex became an important issue. Although around 10 years have passed since the verification of the c-ring rotation, little progress has been made on the rotary dynamics of Fo, compared with F1, owing to challenges in handling the complicated membrane system and difficulty in stably charging the membrane potential high enough to reverse F1. Although detergent-solubilized FoF1 was subjected to the rotation assay in ATP hydrolysis conditions in early studies (64, 65), the observed rotation was insensitive to the gold-standard inhibitor of F1, dicyclohexyl-carbodiimide (DCCD), implying that the observed rotating is not coupled with the proton translocation of Fo (64, 66). The subunit interactions of Fo are weakened in the presence of detergent, which often causes subunit dissociation in biochemical assays (67). Actually, it has been later reported that the rotation in this system is insensitive to mutation at the conserved Arg of the a subunit (68). Verification of the c-ring rotation came from biochemical experiments showing that crosslinkage of the c-ring with the rotor subunits of F1 (γ and ε subunits) does not diminish ATP synthesis activity (69), while the a–c crosslink abolishes ATPase activity coupled with proton translocation (70). Further verification was made by detection of the exchanged cross-link product between the a and c subunits, which was probed with a 14C-labelled c subunit (71). Single-molecule imaging of rotation under ATP synthesis conditions has also been attempted. The rotation of FoF1 reconstituted in liposome was detected from the dipole moment angle of the fluorescent marker dye incorporated into a rotor subunit (72) or Förster resonance energy transfer (FRET) efficiency between two fluorescent dyes introduced into the stator and rotor subunits (73). A drawback of these experiments is that the membrane potential is transient and, therefore, it is very difficult to correlate the observed rotational velocity with the membrane potential. However, one essential property of Fo rotation was revealed with the FRET experiment: multiple stepping rotation was detected that was interpreted as 36° steps based on the 10-fold symmetry of the rotor (74). The 36° stepping rotation was later proved in the rotation assay under ATP hydrolysis conditions where a gold nanorod was used as the rotation probe (75). FoF1 was reconstituted into a nanodisc of lipid bilayer, and the rotation was monitored from the angle of polarized scattered light along the long axis of the nanorod. However, understanding the dynamics of Fo rotation is still in its early stages. Experimental systems that allow stable charging of the membrane potential simultaneously with observation of F1 rotation with high spatiotemporal resolution are highly awaited.

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Conflict of Interest

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