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Chemomechanical coupling of F_1-ATPase under hydrolysis conditions

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F_1-ATPase (F_1) is the smallest rotary motor protein that couples ATP hydrolysis/synthesis to rotary motion in a highly reversible manner. F_1 is unique compared with other motor proteins because of its high efficiency and reversibility in converting chemical energy into mechanical work. To determine the energy conversion mechanism of F_1-ATPase, we developed a novel single-molecule manipulation technique with magnetic tweezers and determined the timing of P_i release, which was the last unknown piece of the chemomechanical coupling scheme of F_1. The established fundamental chemomechanical coupling scheme provides evidence to explain the high reversibility between catalysis and mechanical work.

Key words: F_1-ATPase, single-molecule biophysics, mechanochemistry, magnetic tweezers

Cells contain various types of molecular motor proteins that couple chemical energy to mechanical work and play important roles in biological activities. F_oF_1-ATP synthase (F_oF_1), described in this review, is a type of motor protein that can synthesize ATP by coupling the rotary motion driven by the "proton motive force (*pmf*)," which comprises the proton gradient and voltage difference across the cell membrane. The F_1 motor is a catalytically important part of F_oF_1 and has the unique ability to reverse the process of energy conversion from chemical to mechanical with high efficiency. Its performance surpasses that of artificial engines; therefore, it has been a longstanding goal of many researchers to understand the operating principle of F_1. In this review, we describe the interesting working principles determined so far for F_1 and then introduce results from our recent study.

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Basic properties of F_1

F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) is the smallest motor protein that couples ATP hydrolysis with rotary motion in a counterclockwise direction when viewed from the cell membrane[1]. In the F_1 molecule, three subunits ($\alpha_3\beta_3\gamma$) function as the smallest component of the rotating system, where $\alpha_3\beta_3$ forms a cylindrical motor stator and the rotor subunit γ penetrates its center[2]. The catalytic sites for ATP hydrolysis are located on each interface between the α and β subunits; thus, there are three sites per F_1 molecule. Moreover, when rotary motions were observed, F_1 hydrolyzed three ATP molecules during one rotation and generated a rotary torque of 40 pNnm rad^{-1}.[3] With respect to energy balance during one revolution, the amount of chemical energy acquired by ATP hydrolysis is approximately equal to the kinetic energy released by rotation. Therefore, F_1 is extremely efficient at energy conversion from chemical to mechanical energy. In addition, F_1 not only rotates in the counterclockwise direction driven by ATP hydrolysis but also synthesizes ATP from ADP and inorganic phosphate (P_i) when the rotor is forcibly rotated in the reverse direction[4,5]. Thus, F_1 can achieve the reversible chemomechanical energy conversion. Therefore, to further understand this unique energy conversion mechanism that is highly efficient and reversible, much research has been dedicated to completing the chemomechanical coupling scheme at elementary-step resolution.

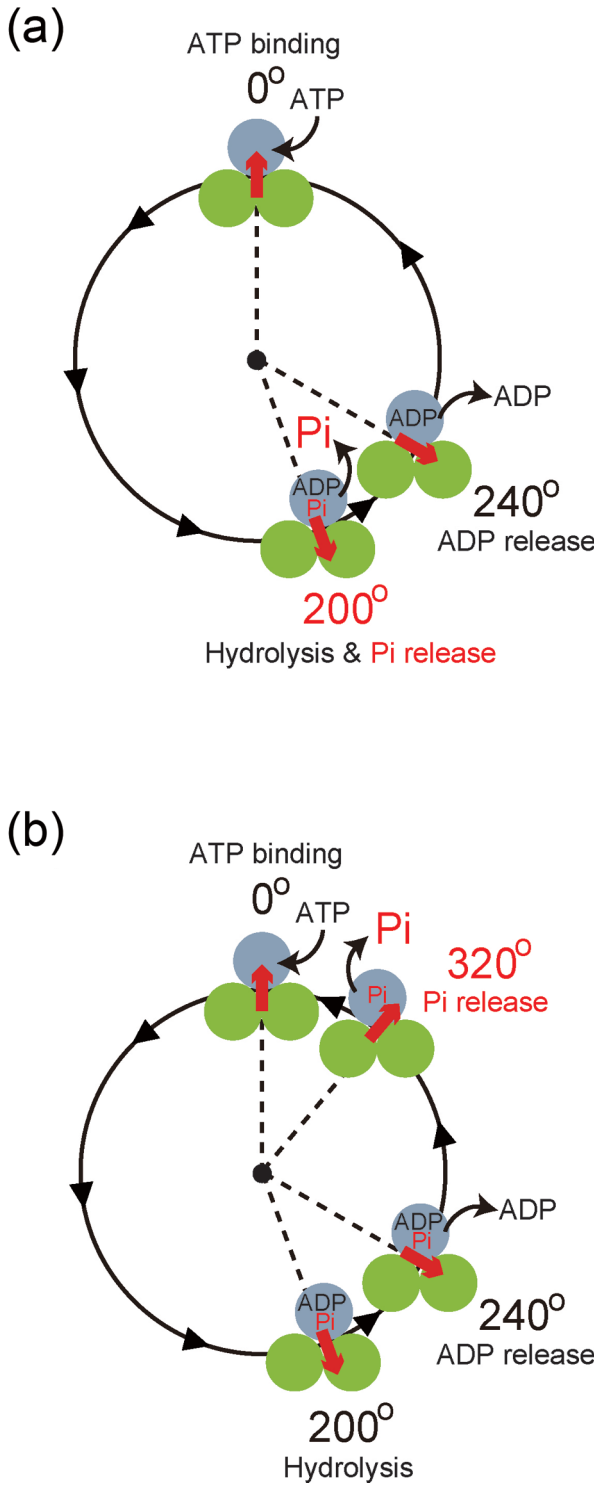

Figure 1 Models of P_i release in chemomechanical coupling of F_1. The circles and red arrows represent the catalytic state of the β subunits and the angular positions of the γ subunit. Among the elementary steps of ATP hydrolysis, the coupling scheme of ATP binding, hydrolysis, and ADP release have already been elucidated. There are two possible models for P_i release: (a) the 200°- and (b) 320°-release models.

Reaction scheme of F_1

It is widely accepted that F_1 tightly couples its catalysis with mechanical work[4,6,7], and therefore, the chemical state of the catalytic sites can be detected by observing the rotary motion at the single molecular level. Indeed, the orientation of F_1 rotor subunits at each elementary step of ATP hydrolysis (i.e., the reaction angle) has been identified using single-molecule observation, and the chemomechanical coupling scheme of F_1 under hydrolysis conditions is almost complete (Fig. 1)[3,6,8–10]. In this scheme, the three catalytic sites of F_1 shift the reaction phase by ±120° from each other, and they follow the same reaction pathway and cooperatively rotate the γ subunit in one direction. Focusing on the reaction pathway of one catalytic site, the bound ATP is hydrolyzed into ADP and P_i after γ rotates 200° from the ATP binding angle[11]. In addition, the produced ADP is released from the catalytic site after another approximately 40° rotation[8,9]. With respect to the reaction angle for P_i release, results from some studies have provided two potential possibilities: the same angle (Fig. 1a) or 120° apart (Fig. 1b) from the angle for ATP hydrolysis. However, the angle has not yet been unambiguously identified[10,12] because it is difficult to measure the dissociation of P_i from one particular catalytic site of the three because of its fast dissociation rate (k=~1000 s^{-1})[8,10,13]. Nonetheless, a large free energy difference between the P_i-bound state and no-bound state of the catalytic sites (i.e., a large amount of free energy change due to P_i release) has been suggested from conventional biochemical studies; therefore, P_i release is considered to play an important role in rotary motion[14]. In our recent study, we built an experimental system to reveal the progress of catalysis from the response after single-molecule manipulation and attempted to clarify it[13].

Single-molecule manipulation of F_1-ATPase

As mentioned earlier, F_1 tightly couples a chemical reaction to rotary motion; therefore, we were able to observe the prolonged rotational pauses due to the hydrolysis reaction intermediate (τ=300 ms) when we used the β^{E190D} F_1 mutant, which significantly slows down ATP hydrolysis activity[9]. During this prolonged pause, we switched on the magnetic tweezers and stalled the rotor part of F_1 conjugated to a magnetic bead at the pausing angle, which corresponds to the reaction angle of ATP hydrolysis (200°). After a certain time had elapsed, we switched off the magnetic tweezers and observed the behavior of F_1 right after release from the stall (Fig. 2a). Our data indicated that F_1 showed two types of behavior (Fig. 2b, c):

i) rotated forward to the next pausing angle (referred to as "ON");

ii) stayed at the original pausing angle (referred to as "OFF").

The behavior of "ON" indicates that ATP hydrolysis has

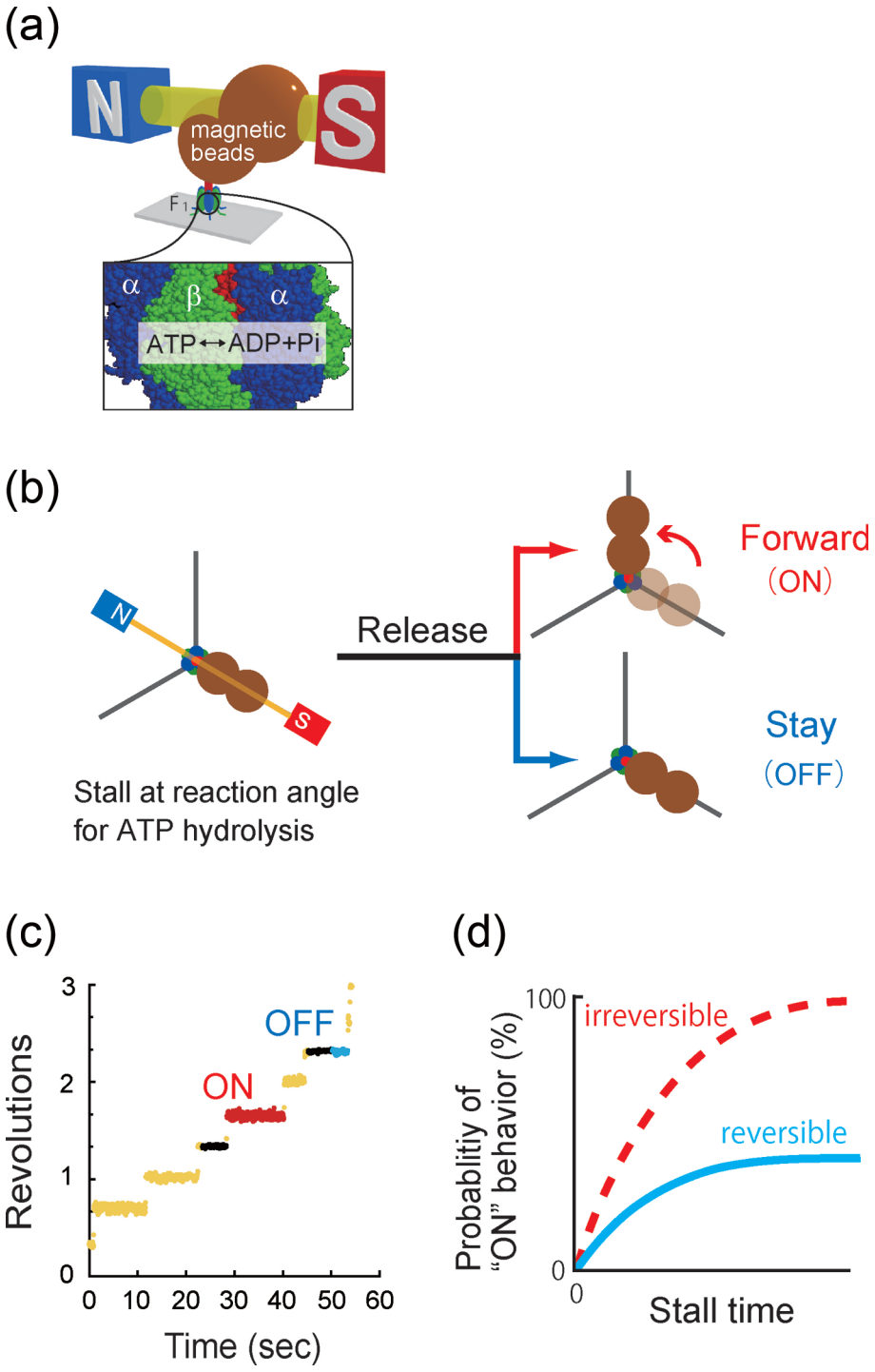

Figure 2 Measurement of chemical equilibrium between hydrolysis and synthesis by single-molecule manipulation. (a) Schematic image of the experimental setup with magnetic tweezers. The probe for detecting the rotary motion and manipulation with magnetic tweezers (i.e., magnetic beads) is immobilized on the top of the rotor subunit of F_1. (b) Experimental procedure of the stalling experiment for the detection of ATP hydrolysis progress. When F_1 pauses owing to the reaction intermediate state of ATP hydrolysis, the magnetic tweezers are switched on to stall F_1 at the reaction angle for ATP hydrolysis (i.e., 200°) and then switched off to release the motor after the set period has lapsed. The released motor shows two behaviors: rotating forward ("ON") or staying at the original pausing angle ("OFF"), indicating that ATP hydrolysis is or is not complete, respectively. (c) The time course of the stalling experiment where stalling was performed twice (black periods). In the first trial, F_1 rotated forward to the next pausing angle immediately after the release ("ON"). In the second trial, F_1 stayed at the original pausing angle ("OFF"). (d) Expected results of the stalling measurements. When hydrolysis is irreversible, the probability of the occurrence of the "ON" behavior increases to 100% as the stall time becomes longer. In contrast, when hydrolysis is reversible, the probability converges to a certain value less than 100%.

been completed during the stall, whereas that of "OFF " indicates that ATP hydrolysis has not yet been completed because F_1 tightly couples the catalysis to the rotary motion. Accordingly, we can determine whether ATP hydrolysis has been completed by analyzing the behavior of F_1.

When P_i is released at the same angle as ATP hydrolysis (i.e., 200°), the produced P_i is released immediately after ATP hydrolysis, and accordingly, F_1 can no longer synthesize ATP (Fig. 1a). In other words, the hydrolysis reaction becomes irreversible because of P_i release, which results in F_1 showing the "ON" behavior almost 100% of the time when the stall is longer than the reaction time constant of ATP hydrolysis (red line in Fig. 2d). In contrast, when P_i is released at the angle where the rotor rotates 120° from the reaction angle of ATP hydrolysis (i.e., 320°), the produced P_i cannot be released and F_1 can resynthesize ATP during the stall even if ATP hydrolysis has been completed (Fig. 1b). In other words, even after being stalled for a long time, the ATP hydrolysis step is reversible; therefore, the probability of occurrence of the "ON" behavior should converge to a constant value depending on the equilibrium level between hydrolysis and synthesis (blue line in Fig. 2d). Here, we consider the actual measurements. When we focused on the data for stalling times <3 s, the probability of "ON" (i.e., the probability of hydrolysis completion) increased depending on the stalling time and reached a plateau level of 70% (Fig. 3b). This result, which corresponds to the time course of hydrolysis to reach chemical equilibrium, showed convergence to a constant value, and accordingly, the reversibility of the hydrolysis step was maintained even after stalling with magnetic tweezers. Next, we focused on the data for stalling times>3 s; the probability of hydrolysis completion slowly and gradually increased toward 100% (Fig. 3a). These data suggest that the hydrolysis step became irreversible because of slow P_i release from the catalytic site at equilibrium; thus, it represents the time course of P_i release. From the analysis of this increment, we determined the dissociation rate of P_i at 200° to be 0.021 s^{-1}, which was very slow when compared with the dissociation rate during free rotation (k=~1000 s^{-1})[8,10,13]. From the discrepancy of the kinetic values, the possibility of P_i release at 200° was eliminated and the timing of P_i release as 320° (Fig. 1b) was identified accordingly, which completed the chemomechanical coupling scheme of F_1 under the hydrolysis conditions. In addition, when we added P_i into the solution, the probability of "ON" decreased to approximately 70% depending on its concentration but did not change further (Fig. 3a). If P_i release immediately after hydrolysis is essential for rotary motion, F_1 cannot rotate until P_i is dissociated even if hydrolysis has been completed. In other words, the probability of "ON" should decrease to 0% depending on the concentration of P_i because addition of P_i prevents its dissociation from the catalytic site. However, the actual probability converged to approximately 70%, which shows that F_1 can drive the rotation even if P_i has not been dissociated right after

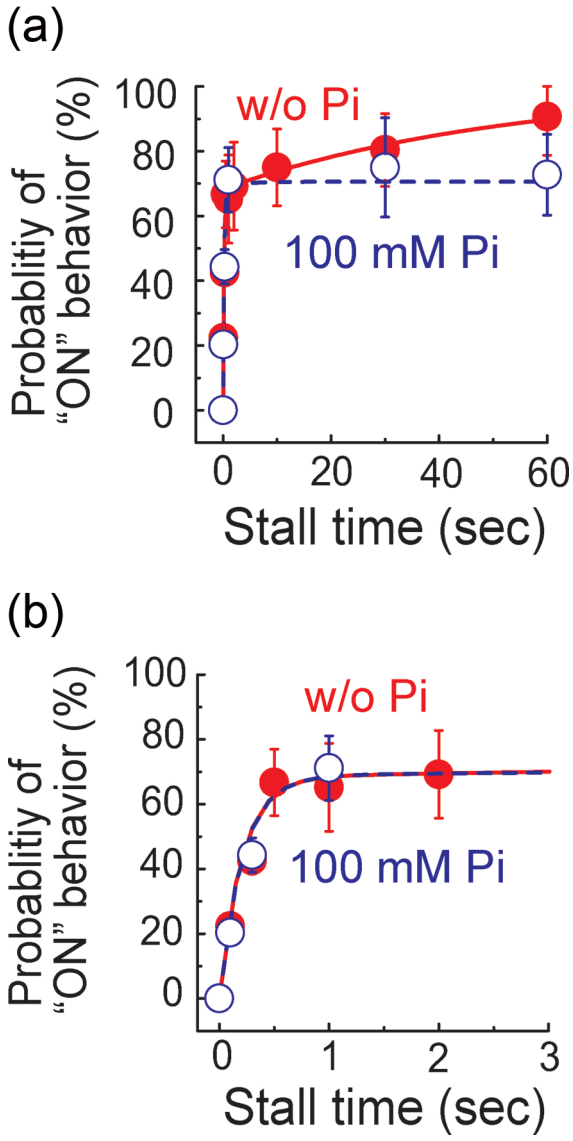

Figure 3 Experimental results of chemical equilibrium between hydrolysis and synthesis. (a) The time course of the probability of the occurrence of the "ON" behavior in the presence (blue) or absence (red) of inorganic phosphate. (b) A close-up from 0 to 3 s.

hydrolysis at 200°. Thus, P_i release right after hydrolysis is found to be a side reaction which rarely occurs in a physiological condition, and we confirmed the timing of P_i release as 320°.

P_i binding and ATP synthesis

In the completed chemomechanical coupling scheme under the hydrolysis conditions, the P_i product was found to dissociate from the catalytic site later than ADP, the other product of hydrolysis (Fig. 1b). In fact, among the other molecular motors driven by ATP hydrolysis, P_i is thought to dissociate from the catalytic site before ADP[15,16]. Here, we consider the specific function of F_1 (i.e., ATP synthesis)

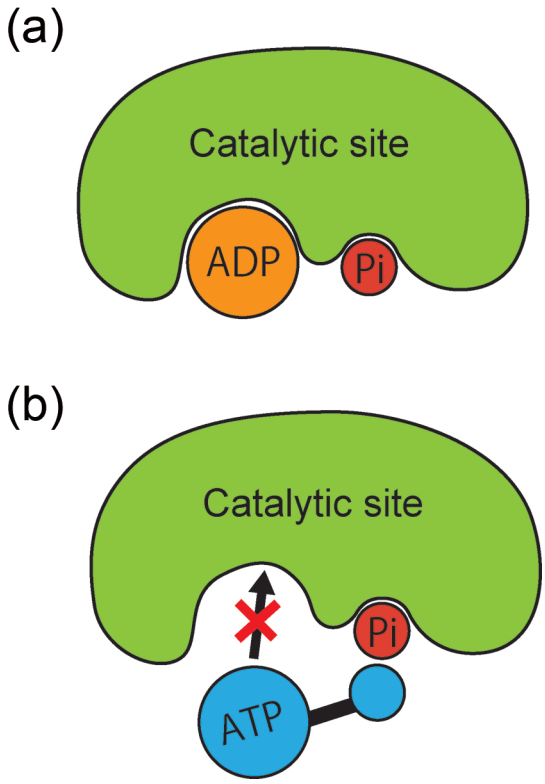

Figure 4 Role of inorganic phosphate in ATP synthesis. Inorganic phosphate (P_i) should be bound to the catalytic site before ATP or ADP binding. The P_i-bound state does not prevent ADP binding (a) but prevents ATP binding (b) due to steric hindrance.

from the viewpoint of the differences in the order of product dissociation. In vivo, the F_o molecular motor, which is the other motor that makes up F_oF_1, applies some external force on F_1 and enables F_1 to synthesize almost all ATP molecules required for living activities. ATP is 10-fold more abundant than ADP in cells[17]; therefore, mechanisms for selective ADP-binding as a substrate from a large pool of ATP is essential for efficient ATP synthesis. When considering the reaction scheme under ATP synthesis conditions, which is hypothesized to be the reverse of that under the hydrolysis conditions mentioned earlier, we found that P_i binds to the catalytic site before ADP. This binding would be advantageous for the selective binding of ADP during ATP synthesis because P_i competitively prevents ATP from binding to the catalytic site because of electrostatic repulsion or steric hindrance but does not prevent ADP binding (Fig. 4). Previous studies have shown that P_i is 10-fold more abundant in cells than ATP, and the affinity of P_i for the catalytic site is much higher than that of ATP as γ rotates in the synthesis direction (clockwise direction)[10,13,18]. Therefore, because of the specific chemomechanical coupling scheme, F_1 can selectively bind to ADP in the presence of a large pool of ATP in cells and synthesize ATP with high efficiency.

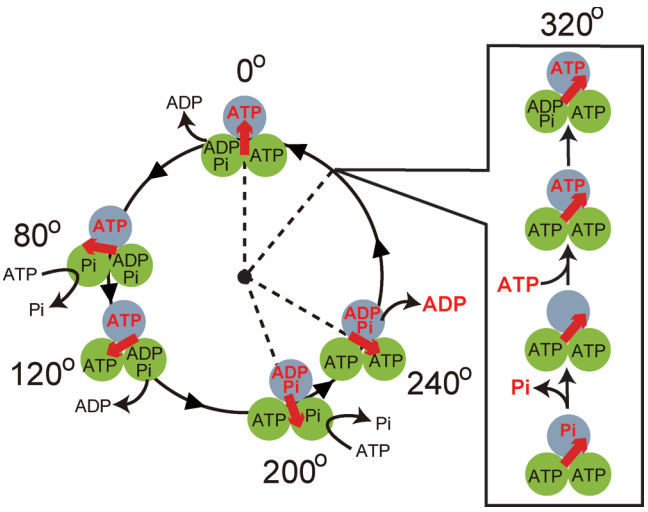

Figure 5 Chemomechanical coupling scheme under hydrolysis conditions. A model for the scheme at high ATP concentrations. ATP binds to the catalytic site at the 320° state immediately after P_i release and before relatively slow hydrolysis on another catalytic site at the 200° state; all the three catalytic sites are occupied by nucleotides.

Remaining questions on the reaction scheme

In the established chemomechanical coupling scheme, two of the three catalytic sites bind to nucleotides and the remaining one binds to P_i at the 320° state. In contrast, a recent biochemical study identified that three nucleotides were bound to catalytic sites at high ATP concentrations[19]. In addition, the crystal structure of F_1, whose catalytic sites are occupied by three nucleotides, was elucidated by x-ray structure analysis[20]; results from both studies seem to be inconsistent with our scheme. Here, we have formulated a new model to resolve these conflicts (Fig. 5). In this model, at high ATP concentration, the catalytic site at the 320° state releases P_i and becomes empty, whereas another catalytic site hydrolyzes ATP. Then, before hydrolysis completion, another ATP binds to the empty catalytic site; thus, the number of bound nucleotides increases to three (Fig. 5). Of the models we considered, this one seems to be the most likely. However, it may not be completely correct because it is based on the hypothesis that rotary motion cannot proceed until hydrolysis is complete even if the main torque-generating steps, such as P_i release and ATP binding[10,13,18], have been completed. To address this discrepancy, it is necessary to simultaneously measure the timing of rotation and P_i release, which we hope to complete in the near future.

Future prospects

Single-molecule observation and manipulation techniques have enabled us to detect the chemical state of the F_1 catalytic sites. As such, we were able to identify the timing of P_i release and complete the chemomechanical coupling scheme under hydrolysis conditions. In the future, it is de-

sirable to precisely understand the coupling scheme under synthesis conditions, which is the physiological role of F_1. To accomplish this, it is necessary to comprehensively measure the reaction rate constants in the synthesis direction using the single-molecule manipulation technique covered in this review or to visualize the rotary motion of F_oF_1 driven by the *pmf*. If the coupling scheme under synthesis conditions was determined, our aim would be to determine the universal operating principles that govern molecular motor proteins that couple chemical reactions and mechanical motion.

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