Chemomechanical coupling of F$_1$-ATPase under hydrolysis conditions

Rikiya Watanabe$^1$ and Hiroyuki Noji$^1$

$^1$Department of Applied Chemistry, School of Engineering, The University of Tokyo, Bunkyo-ku, Tokyo 113-8656, Japan

Received January 13, 2012; accepted March 15, 2012

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$_1$F$_0$-ATP synthase (F$_1$F$_0$), 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$_1$F$_0$, 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.

Basic properties of F$_1$

F$_1$ ($\alpha_3\beta_3\gamma\delta\epsilon$) 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 ($\alpha\beta\gamma$) function as the smallest component of the rotating system, where $\alpha\beta\gamma$ forms a cylindrical motor stator and the rotor subunit $\gamma$ penetrates its center$^2$. The catalytic sites for ATP hydrolysis are located on each interface between the $\alpha$ and $\beta$ 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.
It is widely accepted that $F_1$ tightly couples its catalysis with mechanical work\textsuperscript{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)\textsuperscript{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 $\gamma$ subunit in one direction. Focusing on the reaction pathway of one catalytic site, the bound ATP is hydrolyzed into ADP and $P_i$ after $\gamma$ rotates 200° from the ATP binding angle\textsuperscript{11}. In addition, the produced ADP is released from the catalytic site after another approximately 40° rotation\textsuperscript{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\textsuperscript{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 ($k=\sim1000\text{ s}^{-1}$)\textsuperscript{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\textsuperscript{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\textsuperscript{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 ($r=300\text{ ms}$) when we used the $\beta_{E190D}$ $F_1$ mutant, which significantly slows down ATP hydrolysis activity\textsuperscript{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 F₁. (b) Experimental procedure of the stalling experiment for the detection of ATP hydrolysis progress. When F₁ pauses owing to the reaction intermediate state of ATP hydrolysis, the magnetic tweezers are switched on to stall F₁ 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, F₁ rotated forward to the next pausing angle immediately after the release (“ON”). In the second trial, F₁ 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 F₁ tightly couples the catalysis to the rotary motion. Accordingly, we can determine whether ATP hydrolysis has been completed by analyzing the behavior of F₁.

When Pᵢ is released at the same angle as ATP hydrolysis (i.e., 200°), the produced Pᵢ is released immediately after ATP hydrolysis, and accordingly, F₁ cannot release ATP (Fig. 1a). In other words, the hydrolysis reaction becomes irreversible because of Pᵢ release, which results in F₁ 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 Pᵢ is released at the angle where the rotor rotates 120° from the reaction angle of ATP hydrolysis (i.e., 320°), the produced Pᵢ cannot be released and F₁ 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 Pᵢ release from the catalytic site at equilibrium; thus, it represents the time course of Pᵢ release. From the analysis of this increment, we determined the dissociation rate of Pᵢ 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 Pᵢ release at 200° was eliminated and the timing of Pᵢ release as 320° (Fig. 1b) was identified accordingly, which completed the chemomechanical coupling scheme of F₁ under the hydrolysis conditions. In addition, when we added Pᵢ into the solution, the probability of “ON” decreased to approximately 70% depending on its concentration but did not change further (Fig. 3a). If Pᵢ release immediately after hydrolysis is essential for rotary motion, F₁ cannot rotate until Pᵢ is dissociated even if hydrolysis has been completed. In other words, the probability of “ON” should decrease to 0% depending on the concentration of Pᵢ because addition of Pᵢ prevents its dissociation from the catalytic site. However, the actual probability converged to approximately 70%, which shows that F₁ can drive the rotation even if Pᵢ has not been dissociated right after hydrolysis at 200°. Thus, Pᵢ release right after hydrolysis is found to be a side reaction which rarely occurs in a physiological condition, and we confirmed the timing of Pᵢ release as 320°.

Pᵢ binding and ATP synthesis

In the completed chemomechanical coupling scheme under the hydrolysis conditions, the Pᵢ 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, Pᵢ is thought to dissociate from the catalytic site before ADP 15,16. Here, we consider the specific function of F₁ (i.e., ATP synthesis)
Watanabe and Noji: Rotary mechanism of F\textsubscript{1}-ATPase from the viewpoint of the differences in the order of product dissociation. In vivo, the F\textsubscript{o} molecular motor, which is the other motor that makes up F\textsubscript{o}F\textsubscript{1}, applies some external force on F\textsubscript{1} and enables F\textsubscript{1} to synthesize almost all ATP molecules required for living activities. ATP is 10-fold more abundant than ADP in cells\textsuperscript{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\textsubscript{i} binds to the catalytic site before ADP. This binding would be advantageous for the selective binding of ADP during ATP synthesis because P\textsubscript{i} competes to prevent 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\textsubscript{i} is 10-fold more abundant in cells than ATP, and the affinity of P\textsubscript{i} for the catalytic site is much higher than that of ATP as γ rotates in the synthesis direction (clockwise direction)\textsuperscript{10,13,18}. Therefore, because of the specific chemomechanical coupling scheme, F\textsubscript{1} can selectively bind to ADP in the presence of a large pool of ATP in cells and synthesize ATP with high efficiency.

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

**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\textsubscript{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\textsubscript{i} at the 320° state. In contrast, a recent biochemical study identified that three nucleotides were bound to catalytic sites at high ATP concentrations\textsuperscript{19}. In addition, the crystal structure of F\textsubscript{1}, whose catalytic sites are occupied by three nucleotides, was elucidated by x-ray structure analysis\textsuperscript{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\textsubscript{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\textsubscript{i} release and ATP binding\textsuperscript{10,13,18}, have been completed. To address this discrepancy, it is necessary to simultaneously measure the timing of rotation and P\textsubscript{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\textsubscript{1} catalytic sites. As such, we were able to identify the timing of P\textsubscript{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 $F_1$ 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.

**Acknowledgment**

The authors thank all the members of the Noji Laboratory.

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