Characterization of the temperature-sensitive reaction of F₁-ATPase by using single-molecule manipulation

Rikiya Watanabe¹² & Hiroyuki Noji¹

¹Department of Applied Chemistry, School of Engineering, The University of Tokyo, Bunkyo-ku, Tokyo 113-8656, Japan, ²PRESTO, JST, Bunkyo-ku, Tokyo 113-8656, Japan.

F₁-ATPase (F₁) is a rotary motor protein that couples ATP hydrolysis to mechanical rotation with high efficiency. In our recent study, we observed a highly temperature-sensitive (TS) step in the reaction catalyzed by a thermophilic F₁ that was characterized by a rate constant remarkably sensitive to temperature and had a Q₁₀ factor of 6–19. Since reactions with high Q₁₀ values are considered to involve large conformational changes, we speculated that the TS reaction plays a key role in the rotation of F₁. To clarify the role of the TS reaction, in this study, we conducted a stall and release experiment using magnetic tweezers, and assessed the torque generated during the TS reaction. The results indicate that the TS reaction generates the same amount of rotational torque as does ATP binding, but more than that generated during ATP hydrolysis. Thus, we confirmed that the TS reaction contributes significantly to the rotation of F₁.
with an aspartic acid\textsuperscript{26,27}. Glu-190 of the \(\beta\)-subunit of TF₃ has been identified as a critical residue in ATP hydrolysis\textsuperscript{28–30}, and is termed the “general base” since this residue seems to induce an in-line attack of the water molecule on the \(\gamma\) phosphate and initiate the hydrolysis reaction by activating the water molecule. Another single molecule study revealed that this new reaction intermediate occurs at the angle where the \(\beta\) subunit waits for ATP binding (0° in Fig. 1).\textsuperscript{26} Although this reaction has not been further characterized, the rate constant was found to be remarkably sensitive to temperature. The rate constant increased by a factor of 6–19 for every 10°C rise in temperature\textsuperscript{26–28} (\(Q_{10} = 6–19\)), which was unusually high compared to conventional \(Q_{10}\) values of around 2. In general, reactions with high \(Q_{10}\) values involve large conformational changes. Therefore, this reaction may play a key role in rotation and torque generation. Hereafter, this reaction has been referred to as the temperature-sensitive reaction (TS reaction).

To evaluate the torque generated during each step of the reaction, we recently developed a novel method to measure the equilibrium constant of the F₁ reaction at various rotational angles\textsuperscript{21}. Through this method, we arrested F₁ in the transient conformation using magnetic tweezers and observed the behavior of F₁ immediately after release from arrest. From the analysis of the behavior of F₁, we could simultaneously determine the rate constant for each forward and reverse step of the reaction at various rotational angles. Thus, we could measure the equilibrium constant of each step of the reaction. Because the equilibrium constant is a measure of the difference in the free energy of the pre- and post-reaction states, \(\Delta G(0) = -k_BT\ln K_G(0)\), the torque generated during the reaction can be estimated from the derivative of the free energy, \(d\Delta G(0)/d\theta\).

In the present study, we perform a stalling experiment to elucidate how F₁ modulates the rate and equilibrium constants of the TS reaction as a function of the rotational angle and attempt to assess its contribution in torque generation. The results will contribute to understanding the chemomechanical energy coupling of F₁ at the resolution of the elementary reaction step.

**Results**

**Temperature dependence of the rotation of the TF₁(βE190D) mutant.** We observed the rotation of the mutant F₁, namely, \(\alpha\beta(\text{E}190\text{D})\gamma\gamma\), in the presence of 1 mM ATP at 18, 23, and 28°C (Fig. 2a). Between 18 and 28°C, the mutant F₁ rotated with 80° and 40° substeps (Fig. 2b); the rate limiting steps of the 80° and 40° substeps were identified to be the TS reaction and ATP hydrolysis, respectively, in our previous study\textsuperscript{26}. The dwell time prior to the 80° substep (TS dwell) showed a strong dependence on temperature (Fig. 2c). By fitting the histograms with exponential functions, the time constants of the TS reaction at 18, 23, and 28°C were determined to be 330, 96, and 43 ms, respectively (Fig. 2c). In contrast, the dwell time before the 40° substep (hydrolysis dwell) was not dependent on temperature and was determined to be 208 ms for 18°C, 235 ms for 23°C, and 270 ms for 28°C (Fig. 2d). These results were consistent with the results of our previous study on the TS reaction\textsuperscript{26}.

**Manipulation of single F₁ rotation.** For manipulating the rotation of the \(\gamma\) subunit of F₁, a magnetic bead (\(\phi = 200\) nm) was attached to it and the \(\alpha\beta\beta\) ring was immobilized on the glass surface. For the stalling experiments, the rotation of F₁ was observed under conditions under which the TS dwell was lengthened enough to

![Figure 1](image_url)  
**Figure 1** | Reaction scheme of F₁. The circles and red arrows represent the catalytic states of the \(\beta\) subunits and the angular positions of the \(\gamma\) subunit, respectively. Each \(\beta\) subunit hydrolyzes a single molecule of ATP during one turn of \(\gamma\), whereas three \(\beta\) subunits differ the reaction phase by 120°. The catalytic state of the top \(\beta\) subunit (cyan) has been indicated for clarity. ATP binding, TS reaction, hydrolysis, ADP release, and Pi release occur at 0°, 0°, 200°, 240°, and 320°, respectively.

![Figure 2](image_url)  
**Figure 2** | Rotation of mutant F₁ (αβ(Ε190D))γ at various temperatures. (a) Time course of rotation of the mutant F₁ in the presence of 1 mM ATP at 18 (blue), 23 (green), and 28°C (red). (b) Histograms of the angular position during rotation as calculated from Fig. 2a. (c, d) Histograms of the dwell time of the pause prior to the 80° substep (TS dwell) or the 40° substep (hydrolysis dwell). Curves were plotted using a single-order reaction scheme. \(k = \exp(-\frac{kT}{h})\), where \(kT_0^{-\text{obs}}(18°C) = 3.0\) s\(^{-1}\), \(k_{TS}^{-\text{obs}}(23°C) = 10\) s\(^{-1}\), \(k_{TS}^{-\text{obs}}(28°C) = 23\) s\(^{-1}\), \(k_{\text{Hyd}}(18°C) = 4.8\) s\(^{-1}\), \(k_{\text{Hyd}}(23°C) = 4.3\) s\(^{-1}\), and \(k_{\text{Hyd}}(28°C) = 4.6\) s\(^{-1}\).
able recording at approximately 1,000 fps using a mutant F1, αβ(E190D)γ. As mentioned above, we can distinguish between the angular positions for the TS reaction and the hydrolysis by analyzing the TS and hydrolysis dwell times (Figs. 2c, 2d). When F1 paused for the TS reaction, we turned on the magnetic tweezers to arrest F1 at the target angle (Fig. 3a). After the set period had elapsed, we turned off the magnetic tweezers and released F1 from the arrest. Following release, F1 showed one of two behaviors: rotating directly forward to the next catalytic angle (red in Fig. 3b), i.e., skipping the pause at the original ATP-binding angle, or returning to the original ATP-binding angle (blue in Fig. 3b) without exception. Forward rotation of F1 implied that it had completed the TS reaction and exerted a torque on the magnetic beads; backward rotation of F1 meant that it had not completed the TS reaction because it did not catalyze the reaction and hence could not generate a torque. These behaviors of F1 are hereafter referred to as “ON” (forward rotation) and “OFF” (backward rotation), respectively. Using the above-mentioned methodology, we conducted the stalling experiments in the angle range of ±50°, where the standard deviation of the arrested angle was 5.8°. The following sections discuss the analysis of the probability (PON) of ON events against the total trials.

Angular dependence of the kinetic parameters of the TS reaction. Using the mutant F1, experiments were conducted at 18°C, where the TS dwell time was 330 ms (Fig. 2c). Fig. 4a shows PON plotted against the stall time. PON increased with both the stall angle and the stall time (Fig. 4a), which is similar to our previous observation of ATP binding to wild-type F1αβ. In addition, PON did not always saturate to 100% but converged to a certain value, e.g., 60% for +10° stall (green line in Fig. 4a). These observations imply that the TS reaction is reversible, and that reverse reaction also occurs during stalling. Therefore, the plateau level indicates the equilibrium level between the pre- and post-TS reaction states. To confirm the reversibility, we analyzed the behaviors immediately after the OFF events; i.e., dwell times to spontaneously conduct 80° steps (dwell times at 0° in Fig. 1) immediately after the OFF events (blue points in Fig. 3b). Here, to avoid including data from before the equilibrium, only experiments with longer stalling times, in which PON achieved a plateau were analyzed. The dwell time histogram obtained from all the stall angles showed a single exponential decay, providing a rate constant of 1.1 s⁻¹ (bottom panel in Fig. 4b), which corresponded to that obtained for freely rotating F1s, which were not manipulated with magnetic tweezers (Fig. 2c). This correspondence excluded the possibility of any unexpected inactivation occurring during the stalling that might compete with the TS reaction. We also plotted a histogram of the dwell times to conduct 40° steps (dwell times at 80° in Fig. 1) after the ON events (red points in Fig. 3b). This histogram (top panel in Fig. 4b) was also in good agreement with that obtained for freely rotating F1s (Fig. 2d), confirming that the manipulation did not alter the kinetic properties of F1.

By fitting the time courses of PON based on a reversible reaction scheme, F1 ↔ F1*, the rate constants of the TS reaction and its reverse reaction, kTS−1(18°C) and kTS+1(18°C), were determined for each stall angle (Figs. 5a and 5b). kTS−1(18°C) increased exponentially with the stall angle by approximately 6.2 fold per 20°, which was double that reported previously for ATP binding. kTS−1(18°C) at ±0° was evidently slower than that determined for freely rotating F1s. This phenomenon, which is similar to the ATP-binding step, is attributed to thermal agitation rotary fluctuation that occasionally pushes γ forward, accelerating the TS reaction. In contrast, kTS+1(18°C) was almost constant at approximately 0.3 s⁻¹. Therefore, the equilibrium constant of the TS reaction [kTS−1(18°C)/kTS+1(18°C) = KPTS(18°C)] increased 2.2 times from ~10⁻⁰ to ~10⁻³ (blue points in Fig. 5c), which is a steeper angle dependence than that observed for ATP hydrolysis in the previous study.¹ To confirm the strong angle dependence of the TS reaction under a different condition, the stalling experiments were also conducted at 23 and 28°C, where the time constants of the TS reaction were 96 and 43 ms, respectively (Fig. 2c). The time courses of PON showed the same tendency as that observed at 18°C (Figs. 4c, 4e). The reversibility of the TS reaction at 23 and 28°C was also confirmed from the analysis of the dwell time after arrest (Figs. 4d and 4f). The rate and equilibrium constants were determined as mentioned above (Figs. 5a, 5b, and 5c). The equilibrium constants determined for the TS reaction at 23 and 28°C showed essentially the same angle dependence as that at 18°C (red and green points in Fig. 5c). Thus, the strong angle dependence of the TS reaction is inherent in F1, regardless of the temperature.  

Rotational energy potential. We examined the rotational energy potential during TS dwell, i.e., the waiting state for the TS reaction. The probability distribution of γ-orientation during the TS dwell of mutant F1, αβ(E190D)γ, was measured (orange points in Fig. 6a). The probability distributions obtained were then transformed into the rotational energy potential according to the Boltzmann’s Law, ΔG = -kBTln(P/P₀) (orange points in Fig. 6b). The potential determined was fitted to the harmonic function, ΔG = 1/2καθ², where κ is the torsion stiffness. The determined value of stiffness

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**Figure 3** | Single-molecule manipulation of F1. (a). Schematic image of the manipulation procedures. The gray solid and dashed lines represent the ATP-binding and catalytic angles, respectively. When F1 paused due to TS dwell at the ATP-binding angle, the magnetic tweezers were switched on to stall F1 at the target angle and then turned off to release the motor after the set period had elapsed. A released F1 showed forward (ON) or backward (OFF) rotation with respect to the original ATP binding angle. The behavior of F1 indicated whether the TS reaction was completed (in case of ON) or not (in case of OFF). (b). Examples of stalling experiments for the TS reaction at 18°C. During a pause, F1 was stalled at −6.6° from the original pausing angle for 1.0 s and then released. After being released, F1 rotated to the next catalytic angle without any backward rotation, indicating that the TS reaction had been completed by F1 upon release (red). When F1 was stalled at −9.1° for 1.0 s, it rotated back to its original pausing angle, implying that the TS reaction had not been completed (blue).
as well as those of the other reaction steps, that is, ATP binding, hydrolysis, and Pi release, determined in our previous studies\(^{15,31}\), are shown in Fig. 7. Data points are plotted in the angular diagram of the reaction scheme for one β subunit (Fig. 1), where the pause angles for ATP binding, TS reaction, hydrolysis, and Pi release were assigned as 0°, 0°, 200°, and 320°, respectively. The magnitude of rotational torque (N) is determined by the slope of the rotational energy potential in the post-reaction state, \(dU_{\text{post}}(\theta)/d\theta\)\(^{11,32}\). It is very difficult to measure the rotational potential directly in the post-reaction state, \(U_{\text{post}}(\theta)\). Therefore, in our previous studies, we had estimated the torque generated during each reaction step from the angular dependence of the reverse reaction rate, \(\Delta G_{i}(\theta) = -k_{\text{B}}T[\ln(1/(\theta))]\), which is a measure of the energy difference between the transition state and the post-reaction state, \(\Delta G_{i}(\theta) = U_{\text{post}}(\theta) - U_{\text{TS}}(\theta)\). When we assume that the energy level at the transition state, \(U_{\text{TS}}(\theta)\), is a constant, and does not depend on the rotational angle, the derivative of the energy difference responds to the slope of the potential in the post-reaction state (equivalent to the torque), \(d\Delta G_{i}(\theta)/d\theta = dU_{\text{post}}(\theta)/d\theta\). Therefore, we previously estimated the torque generation from the reverse reaction rate based on this assumption with respect to the energy level for the transition state\(^{11,32}\), which has not been verified experimentally so far. In this study, we used the angle dependence of the equilibrium constant, \(K_{i}(\theta)\), which is a more robust approach to estimate the torque generation. The free energy difference between the pre- and post-reaction states can be determined from the angle dependence of the equilibrium constant; \(\Delta G_{i}(\theta) = U_{\text{post}}(\theta) - U_{\text{TS}}(\theta) = k_{\text{B}}T[\ln(K_{i}(\theta))]\). Because \(U_{\text{post}}(\theta)\) was not affected by the elastic component located on the transmission line to the beads\(^4\), and was almost the same for each reaction step (Fig. 6), the derivative of the free energy difference may be regarded as a measure of the slope of the rotational potential in the post-reaction state (equivalent to the torque), \(d\Delta G_{i}(\theta)/d\theta = dU_{\text{post}}(\theta)/d\theta\). Therefore, the slopes of the equilibrium constants in a semi-log plot (Fig. 7) reflect the magnitude of torque generated upon each reaction step. The estimation shows that

**Discussion**

The equilibrium constant of the TS reaction determined in this study, as well as those of the other reaction steps, that is, ATP binding,
the TS reaction has a slope similar to those of ATP binding and Pi release and a steeper slope than that of ATP hydrolysis. This suggests that the contribution of the TS reaction to torque generation is similar to those of ATP binding and Pi release and is much higher than that of ATP hydrolysis, i.e., the TS reaction contributes significantly to the torque generation of F1.

Considering the extremely high temperature dependence of the TS reaction, this reaction may involve a large-scale conformational rearrangement of the catalytic β-subunit when γ is oriented to the angle for ATP binding. Recent single-molecule studies have revealed that the C-terminal region of the β subunit shows a large-scale conformational change at around 0°, which contributes to generating half of the rotational torque, that is, approximately 20 pN·nm rad⁻¹. \textsuperscript{34,35} Our experimental results suggest that the TS reaction contributes greatly to torque generation at around 0°. Therefore, it is probable that the TS reaction is somehow related to the large-conformational change in the C-terminal region of the β subunit at 0°; however, there has been no direct verification so far. To identify the TS reaction, we hope to visualize simultaneously the conformational change in the β subunit and the rotational motion at the temperature, where F1 shows a distinctive pause due to the TS reaction.

Improper ATP hydrolysis due to an alternative catalytic pathway may be another possible reason for the occurrence of the TS reaction. According to this mechanism, Pi release in the β-subunit at the 320° state (cyan circle at 320° in Fig. 1) may drive the rotation of the 40° substep from 320° to 360° (≈0°) without hydrolyzing ATP in another β-subunit at the 200° state (left green circle at 320° in Fig. 1). This may cause the dwell at 0° for waiting the ATP hydrolysis to occur in the aforementioned β-subunit at the 240° state (left green circle at 0° in Fig. 1). In our experimental data, the angle dependence of the rate constants of the TS reaction and its reverse reaction (Fig. 5) was similar to those of ATP hydrolysis and synthesis\textsuperscript{34}. The forward reaction rate was accelerated towards the counterclockwise direction, while the reverse reaction rate was almost constant and did not depend on the rotary angle, although the slopes of angle dependence are different from each other. Therefore, our result suggests that the TS reaction might occur due to improper ATP hydrolysis at the 240° state (left green circle at 0° in Fig. 1). Simultaneous monitoring of the catalytic events, i.e., ATP binding, hydrolysis, and products release, with the rotational motion will provide insights into this mechanism.

Using single-molecule manipulations, we measured the rate and equilibrium constants of F1 in the transient conformational states, which could not be obtained in the conventional single molecule assay. Moreover, from the equilibrium constants determined by single-molecule manipulations, we evaluated the force generated during the elementary reaction steps. Thus, single-molecule manipulation is a powerful tool for understanding the chemomechanical energy coupling mechanism and holds promise for understanding the functioning of other molecular machines.

**Methods**

**Rotation assay.** The mutant form of F1 from thermophilic Bacillus PS3 (TF1), \(\alpha_{1}\beta^{E190D}\gamma^{E190D}\), was prepared as described previously.\textsuperscript{36} To visualize the rotation of F1, the stator region (\(\alpha_{3}\beta_{3}\gamma_{3}\) subunits) was fixed to a glass surface, and magnetic beads (\(\phi\) approximately 0.3 μm; Seradyn, USA) were attached to the rotor (\(\gamma\) subunit), as the probe for monitoring rotation and for further manipulation. The rotation assay was carried out in a 50 mM MOPS-KOH (pH 7.0) buffer containing 50 mM KCl, 5 mM MgCl\(_2\), and 1 mM ATP. Rotating beads were observed under a phase-contrast microscope (IX-70 or IX-71; Olympus, Japan) with a 100× objective lens. The temperature in the room was controlled with a room air conditioner and monitored with a thermometer located on the sample stage of the microscope. The precision of the temperature control was ±1°C.

**Manipulation with magnetic tweezers.** The stage of the microscope was equipped with magnetic tweezers that could be controlled with the custom-made software.

Figure 6 | Rotational energy potential. (a). Probability distributions of angular positions during the dwell. Red and blue points represent our previous results for the ATP binding dwell of wild-type F1 and the hydrolysis dwell of mutant F1, \(\alpha_{1}\beta^{E190D}\gamma^{E190D}\). Orange points represent the experimental result for the TS dwell of mutant F1 measured in this study. The probability distribution was derived from three molecules. (b). Rotational energy potentials determined from probability distribution according to the Boltzmann’s law, \(\Delta G = -k_{B}T \ln(P_{\text{P}})\). The potentials determined were fitted to the harmonic function \(\Delta G = 1/2 \kappa \theta^{2}\), where \(\kappa\) is the torsion stiffness. Stiffness values determined were 80, 75, and 64 pN·nm for the ATP binding of wild-type F1, the TS reaction, and the hydrolysis of mutant F1, respectively.

Figure 7 | Modulation of equilibrium constants upon γ rotation. Modulation of equilibrium constants upon rotation. All data points are plotted along the reaction scheme for one β subunit (Fig. 1), where the angles for ATP binding, TS reaction, hydrolysis, and Pi release are assigned as 0°, 0°, 200°, and 320°, respectively. Red, blue, and green symbols represent the dissociation constant of ATP (\(K_{d}^{\text{ATP}}\)), the inverse values of the equilibrium constant of ATP hydrolysis (1/\(K_{d}^{\text{P}_{i}}\)), and the dissociation constant of Pi (1/\(K_{d}^{\text{Pi}}\)), determined in the previous study.\textsuperscript{34,32,24} Orange symbols represent the inverse values of the equilibrium constant of the TS reaction (1/\(K_{d}^{\text{TS}}\)), determined in this study.
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