Temperature-sensitive reaction intermediate of F₁-ATPase

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INTRODUCTION

F₁-ATPase is a rotary molecular motor that makes 120° stepping rotations, with each step being driven by a single-ATP hydrolysis. In this study, a new reaction intermediate of F₁-ATPase was discovered at a temperature below 4°C, which makes a pause at the same angle in its rotation as when ATP binds. The rate constant of the intermediate reaction was strongly dependent on temperature with a Q₁₀ factor of 19, implying that the intermediate reaction accompanies a large conformational change. Kinetic analyses showed that the intermediate state does not correspond to ATP binding or hydrolysis. The addition of ADP to the reaction mixture did not alter the angular position of the intermediate state, but specifically lengthened the time constant of this state. Conversely, the addition of inorganic phosphate caused a pause at an angle of +80° from that of the intermediate state. These observations strongly suggest that the newly found reaction intermediate is an ADP-releasing step.

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constant of 1 ms. One of the two reactions before the 40° substep was identified as the ATP-hydrolysis step by using a mutant F$_1$-ATPase with a low rate of ATPase and a slowly hydrolysing ATP analogue, ATP$_g$S (Shimabukuro et al., 2003). Thus, the discovery of the reaction intermediate as an intervening pause during the rotation is crucial for showing the mechanochemical coupling mechanism of F$_1$-ATPase. Here, a new reaction intermediate was found in the rotation assay at low temperatures. Hereafter, the rotary angles before the 80° and 40° substeps are referred to as the binding and the catalytic angle, respectively.

**RESULTS**

The rotational rate of F$_1$-ATPase was determined at various temperatures between 23°C and 2°C at a saturating ATP concentration of 1 mM (Fig 1A). A magnetic bead (φ = 0.2 μm) was attached to the γ-subunit as a rotation marker for imaging with a conventional microscope. The magnetic bead also allowed us to reactivate pausing of the F$_1$-ATPase molecules in the ADP-inhibited form when using magnetic tweezers (Hirono-Hara et al., 2001, 2005). Thus, long-term observation of the rotation was allowed even under conditions in which ADP inhibition severely hampered the rotation, such as in the presence of ADP or in buffer- or temperature-changing experiments. The magnetic bead acts as a viscous load for F$_1$-ATPase; therefore, the rotation against viscous friction sets the pace of the overall reaction cycle. As a result, the 2 ms pause at the catalytic angle became undetectable, and F$_1$-ATPase showed smooth rotation at 3–5 Hz between 23°C and 9°C. The slight decrease in the rotational rate can be attributed to the increase in water viscosity at low temperatures, in agreement with the theoretical line based on the temperature dependence of water viscosity (Fig 1A, orange lines). Below 9°C, the rotational rate decreased significantly compared with the theoretical line. The time courses of the rotation at 2°C and 4°C showed distinct intervening pauses at every 120° (Fig 1B,C), showing that at low
temperatures the reaction step that caused the pause became rate limiting. A histogram of the dwell-time between the pauses at 4 °C followed a single exponential decay with a rate constant of 7.2 s⁻¹ (Fig 2D), indicating that a single reaction step determined the pausing time.

The ATPase rate in a bulk solution was also measured. The rotational rate of F₁-ATPase without a viscous probe was calculated as one-third of the bulk ATPase rate, based on the coupling ratio of 3 ATPs per rotation. It was found that the bulk ATPase rate increased slightly after the initiation of the ATPase assay at temperatures below 20 °C. At 4 °C, the maximum rate was 16 s⁻¹, whereas the initial rate was 8.5 s⁻¹. Such acceleration was not observed in the rotational assay. In Fig 1A, rotational rates estimated from the initial and maximum ATPase rates are shown. The breakpoints around 9 °C in the Arrhenius plots of ATPase also indicate that a certain reaction step became a rate-limiting step at low temperature. On the basis of the Arrhenius plots, Q₁₀ factors between 3 °C and 6 °C were found to be 15 and 18 for the initial and maximum ATPase rates, respectively. These findings were essentially consistent with the Q₁₀ factor of 19 for the rotation assay between 2 °C and 4 °C. Furthermore, the estimated rotational rates were closer to the observed rotational rate at lower temperatures, although the former were slightly higher even at 2 °C. Thus, it was shown that a common reaction step limited the rate of the overall reaction cycle in the rotation assay and in the bulk ATPase assay at low temperature. Hereafter, this reaction is referred to as the temperature-sensitive reaction.

Michaelis–Menten curves were determined in the rotation assay at 4 °C and 23 °C and were compared (Fig 2A). The Vₘₐₓ and Kᵣₐₐₚ values at 4 °C were 20 s⁻¹ and 11.7 μM, respectively. These values at 23 °C were 5.6 s⁻¹ and 1.2 μM, respectively, and showed good agreement with the previously reported values for F₁-ATPase with viscous probes (Yasuda et al., 1998; Rondelez et al., 2005). It was clear that the ATP-binding step does not set the overall rotational cycle at 1 mM ATP at 4 °C; thus, the temperature-sensitive reaction is not an ATP-binding reaction. Interestingly, the step size was always 120°, even at 5 μM ATP, near Kᵣₐₐₚ, in which the rate constants of the temperature-sensitive reaction and ATP binding were similar (Fig 2B, C). This suggests that the temperature-sensitive reaction occurs at the binding angle. To confirm this finding, a buffer-changing experiment was carried out in which the ATP₇S solution was replaced with an ATP solution during the rotation of an F₁-ATP molecule. We have shown that F₁-ATPase slowly hydrolyses ATP₇S and transiently pauses at the catalytic angle until the execution of hydrolysis (Shimabukuro et al., 2003). In the presence of 1 mM ATP₇S at 4 °C, F₁-ATPase showed rotation with discrete 80° and 40° substeps (Fig 3A, B). The upper histogram of Fig 3B shows six peaks, each corresponding to the pauses...
before the 40° or 80° substep. After replacement with 1 mM ATP solution, the pauses before the 40° substep disappeared, and the F1-ATPase molecule made a 120° stepping rotation (Fig 3A,B). This shows that the pause before the 40° substep is due to the slow hydrolysis step. This was also supported by the histogram of the dwell-time before the 80° substep, which yielded a rate constant of 5.7 s⁻¹ (supplementary Fig S2 online), which was essentially consistent with the temperature-sensitive reaction rate; the 40° substep yielded a much higher rate constant of 14.5 s⁻¹. Statistical analysis showed that the angle of the pause before the 80° substep at 1 mM ATPγS from the angle of the temperature-sensitive reaction at 1 mM ATP (Δθ₂ in Fig 3B) was +3.1 ± 8.3°, thereby confirming that the pause before the 80° substep was due to the temperature-sensitive reaction. Conversely, the angle of the pause before the 40° substep (Δθ₁) was −38 ± 8.6°. This showed that the temperature-sensitive reaction occurs at an angle of +40° from the catalytic angle—that is, the binding angle. The angle for the temperature-sensitive reaction was further confirmed by changing the temperature during the observation with 1 mM ATPγS (Fig 3D). When the temperature increased from 4°C to 23°C, F1-ATPase still showed the pause before the 40° substep, whereas the pause before the 80° substep disappeared, indicating that this pause was due to the temperature-sensitive reaction (Fig 3E). The angle of the pause before the 40° substep at 4°C was consistent with the ATPγS-hydrolysis angle at 23°C (Δθ₁ in Fig 3E), whereas that of the pause before the 80° substep from the ATPγS-hydrolysis angle (Δθ₂ in Fig 3E) was +42 ± 12° (Fig 3F). Thus, this finding further verified that the temperature-sensitive reaction occurred at the binding angle. Therefore, the temperature-sensitive reaction is not a hydrolysis step.

These experimental results indicate that the temperature-sensitive reaction was either due to ADP or inorganic phosphate (P_i) release. If this was the case, a large amount of ADP or P_i in the solution would be expected to competitively suppress the apparent rate of the temperature-sensitive reaction—that is, the catalytic site that released ADP/P_i would immediately re-bind ADP/P_i from the solution before initiating the rotation. Therefore, the rotation was next observed in the presence of a large amount of ADP or P_i with 1 mM ATP at 4°C. The rotational rate decreased with an increase in ADP or P_i concentration (supplementary Fig S3).
online), thereby yielding $K_{\text{ADP}}$ of 43 $\mu$M and $K_i^{\text{Pi}}$ of 1.8 mM. The $K_{\text{ADP}}$ and $K_i^{\text{Pi}}$ were also determined in the bulk ATPase measurements to be 1.5 $\mu$M and 1.2 mM, respectively (supplementary Fig S3 online). Although the $K_i^{\text{Pi}}$ values were consistent between bulk and rotation assays, the $K_{\text{ADP}}$ value determined from the bulk assay was lower than that determined in the rotation assay. It was likely that the bulk measurement inevitably resulted in a lower ATPase rate, which was affected by ADP inhibition, particularly under high ADP/ATP ratio conditions. Conversely, the single-molecule observation allowed analysis of only catalytically active molecules discriminating from the ADP-inhibited form. Therefore, we believe that the $K_{\text{ADP}}$ determined in the rotation assay is more reliable.

Buffer-changing experiments were carried out to determine how rotation behaviours were altered when suppressed by ADP or Pi in solution. After observing the rotation at 1 mM ATP, a solution containing ADP or Pi was injected into the sample chamber. In the presence of 10 mM ADP with 1 mM ATP, F$_1$-ATPase continued to show a distinct 120° stepping rotation, although the pause became longer (Fig 4A,D). Furthermore, the pausing angles were consistent with those in the absence of ADP, and the angular shift ($\Delta\theta$) was only 1.1 ± 7.8° (Fig 4G). The rotation in the presence of 10 mM ADP and 120 $\mu$M ATP also yielded similar results ($\Delta\theta$ in Fig 4H was −0.5 ± 7.6°). Thus, these results show that F$_1$-ATPase rebinds and releases ADP at the binding angle. Conversely, when 600 mM Pi was added with 1 mM ATP, the pausing angle became less distinct (supplementary Fig S4 online). To clarify the difference in the pausing angles between the cases with and without Pi, buffer-changing experiments were carried out at a lower ATP concentration of 10 $\mu$M. Fig 4F clearly shows that the pausing angle shifted from the binding angle in the presence of 600 mM Pi. Statistical analysis (Fig 4I) showed that $\Delta\theta$ was −29 ± 9.2° from the binding angle. Thus, it was clarified that Pi re-binding/release occurs at the catalytic angle.

**DISCUSSION**

Our experiments show that the temperature-sensitive reaction is neither an ATP-binding nor ATP-hydrolysis reaction, based on the observation that the temperature-sensitive reaction occurs at the binding angle and the rate constant is independent of ATP concentration. Competitive inhibition experiments with Pi showed that Pi caused a pause at the catalytic angle. Similar experimental data were obtained from high-speed imaging of the γ-subunit rotation at 23°C (Adachi et al., 2007). Thus, Pi release occurs at the catalytic angle and is not relevant to the temperature-sensitive reaction. Conversely, ADP specifically suppressed the apparent rate constant of the temperature-sensitive reaction. Our previous study also showed that the addition of ADP to the solution prolonged the pausing time at the binding angle at 25°C (Muneyuki et al., 2007). These results suggest that the temperature-sensitive reaction might be an ADP release reaction. However, it is possible that the temperature-sensitive reaction is merely a conformational rearrangement step, which is not relevant to any catalytic step. Furthermore, some types of F$_1$-ATPase are reported to be unstable at low temperature (Williams et al., 1984); therefore, one can argue that the pause of the temperature-sensitive reaction could be a result of cold denaturation, which would cause the subunit dissociation. We assessed this possibility by analysing the stability of F$_1$-ATPase using size-exclusion chromatography and by the rotary fluctuation of F$_1$-ATPase during the pause. However, these analyses showed no significant differences at 4°C or 23°C (supplementary Figs S5, S6 online). These data show that the temperature-sensitive reaction cannot be attributed to cold denaturation. We also tested a kinetic model, which assumes that the temperature-sensitive reaction and ADP release are different reaction steps but both occur at the binding angle (supplementary Fig S1 online). However, fitting curves according to this model could not reproduce the distribution of the dwell-time between 120° steps in the presence of 2, 5 and 10 mM ADP with 1 mM ATP. Conversely, all the experimental data were well fitted with simulation curves based on a model in which the temperature-sensitive reaction is assumed to be ADP release. Thus, it is most probable that the temperature-sensitive reaction is the ADP release.

Recently, it was observed that a fluorescent-labelled nucleotide was released from F$_1$-ATPase when the γ-subunit rotated 240° after the binding of fluorescent-labelled ATP to F$_1$-ATPase at 0°C (Adachi et al., 2007). Although this result was obtained in the experiment in which a fluorescent-labelled ATP was used as a substrate at very low concentrations, the result is consistent with our finding that ADP release occurs at the binding angle. This strongly indicates that ADP dissociates at 240° after it binds to F$_1$-ATPase as ATP at 0°C. Our finding that Pi is released at the catalytic angle is also consistent with the result reported by Adachi et al. (2007). However, these studies do not provide information on the angular displacement of γ until Pi dissociates once it binds to F$_1$-ATPase as ATP. With regard to this point, a single-molecule study using a chimera F$_1$-ATPase showed that a certain reaction occurs at 320° after the binding of a nucleotide to F$_1$-ATPase (Ariga et al., 2007). Therefore, Pi release is a strong candidate for the reaction at 320°. On the basis of the above-mentioned points, we propose a possible reaction scheme of the rotation and catalytic reaction (Fig 5). In this scheme, two catalytic sites are occupied with nucleotides, except for the transient full occupation at the ATP-binding step. However, it should be noted that strong evidence is available that proves that the three catalytic sites are always occupied at high ATP concentrations (Weber & Senior, 1997). Therefore, further experiments are required to clarify this apparent discrepancy.

A prominent characteristic of the temperature-sensitive reaction is its strong temperature dependency. The $Q_{10}$ factor of the temperature-sensitive reaction was found to be 19. This was unusually high compared with conventional $Q_{10}$ values of around 2. In general, it is considered that reactions with higher $Q_{10}$ values involve large conformational changes. For example, in myosin, which involves large conformational dynamics, a very high $Q_{10}$ factor of more than 70 was found (Anson, 1992). The assumption of the temperature-sensitive reaction being an ADP-release reaction is reasonable, because the ADP-release step is expected to be coupled with a large conformational change in the β-subunit. The large $Q_{10}$ factor of the temperature-sensitive reaction also explains why the temperature-sensitive reaction was not detected in the high-speed imaging of the rotation (Yasuda et al., 2001). The rate of the temperature-sensitive reaction at 24°C was estimated to be 6.6 × 10$^{-3}$ s$^{-1}$ from the $Q_{10}$ factor. This rate is too fast to be detected even with high-speed imaging.
In their study, the $Q_{10}$ factor of ATPase of *E. coli* F1-ATPase was estimated to be around 2 (refer to Fig 4; Al-Shawi et al., 1997). The ATPase of *E. coli* F1-ATPase was measured near its growth temperature (37°C); by contrast, in our study, the assay was carried out at temperatures much lower than 65°C, which was the growth temperature of *Bacillus* PS3, the source of F1-ATPase. Fig 1A also shows the $Q_{10}$ factor of 2–4 from the ATPase rate at 9°C to 31°C. On the basis of the above-mentioned points, experiments at a considerably low temperature would be required to detect the temperature-sensitive reaction of *E. coli* F1-ATPase.

**METHODS**

**Rotation assay.** F1-ATPase derived from thermophilic *Bacillus* PS3 was prepared, and the rotation assay was carried out as reported previously (Rondelez et al., 2005). The rotation of magnetic beads (≈0.2 μm, Seradyn Inc, Indianapolis, IN, USA) attached to γ-subunit was observed using bright-field or phase-contrast microscopy and manipulated with magnetic tweezers for reactivation of ADP-inhibited F1-ATPase (Hirono-Hara et al., 2005). The images were recorded at a video rate of 30 frames per second. Except for the experiment at 23°C, the microscope with a temperature control system (TOKAI HIT) was set up in a cold room. The temperature was monitored using a thermocouple probe attached to the sample chamber. The precision of the temperature control was ±0.5°C and ±1°C for temperatures below and above 5°C, respectively.

**Measurement of ATP-hydrolysis activity in solution.** ATP-hydrolysis activity of F1-ATPase in solution was measured using an ATP-regenerating system, as reported previously (Rondelez et al., 2005). The initial hydrolysis rate (0–5 s after initiation of the reaction) or the maximum hydrolysis rate was determined from the slope of decrease in absorbance at 340 nm. The time point at which the
maximum rate was attained after initiation of the reaction varied for each temperature. However, as the rate constant of the activation (0.017 s⁻¹ at 4 °C) was considerably slower than the response rate of the ATP-regenerating system (0.4 s⁻¹ at 4 °C), the activation of ATPase could not be due to the ATP-regenerating system. The temperature of the reaction mixture was controlled by placing the cuvette holder in a water bath, and the temperature was measured using a thermocouple probe that was inserted in the reaction cuvette holder in a water bath, and the temperature was measured using a thermocouple probe that was inserted in the reaction mixture. The precision of the temperature control was ±0.5 °C.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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