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Temperature-sensitive reaction intermediate of F_1-ATPase

Rikiya Watanabe[1], Ryota Iino[2], Katsuya Shimabukuro[3], Masasuke Yoshida[4] & Hiroyuki Noji[2+]

[1]Department of Mechanical Engineering, University of Tokyo, Tokyo, Japan, [2]Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka, Japan, [3]Florida State University, Tallahassee, Florida, USA, and [4]Chemical Resources Laboratory, Tokyo Institute of Technology, Yokohama, Japan

F_1-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 F_1-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 Q_{10} 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.

Keywords: ATP synthase; single-molecule observation; F_1-ATPase; temperature dependency

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INTRODUCTION

F_1-ATPase is the water-soluble part of ATP synthase and a rotary molecular motor protein driven by ATP hydrolysis (Junge *et al*, 1997; Dimroth *et al*, 2006). The bacterial F_1-ATPase comprises five subunits—α, β, γ, δ and ε—with a molar ratio of 3:3:1:1:1. The minimum stable ATPase complex is the $\alpha_3\beta_3\gamma$ subcomplex, which is hereafter referred to as F_1-ATPase. The first solved crystal structure of F_1-ATPase showed that the rotor subunit γ was surrounded by the $\alpha_3\beta_3$ stator ring in which the three α- and β-subunits were arranged alternately (Abrahams *et al*, 1994). The catalytic sites were located at the three α–β interfaces, mainly on the β-subunits. Each catalytic site was found to be in a different reaction state: the first site was not occupied by a ligand, the second site bound to the ATP analogue AMP-PNP and the third site bound to ADP, which was later shown to bind to AMP-PNP in the absence of azide (Bowler *et al*, 2007). Although there is some inconsistency (Weber & Senior, 1997), these structural characteristics are basically consistent with the rotary catalytic mechanism (Boyer, 1993), which assumes that each catalytic site is always in a different reaction state, and that the interconversion of these reaction states is accompanied by the mechanical rotation of the γ-subunit. Following the evidence from biochemical and spectroscopic studies (Duncan *et al*, 1995; Sabbert *et al*, 1996), the rotary motion of the γ-subunit in F_1-ATPase was finally confirmed by single-molecule observation (Noji *et al*, 1997).

Since the establishment of the single-molecule rotation assay, attempts have been made to resolve the rotary motion of F_1-ATPase into discrete steps in order to clarify how the rotation is coupled with the elementary reaction steps of ATP hydrolysis. The stepping rotation was first observed under substrate-limiting conditions (Yasuda *et al*, 1998). Under such conditions, F_1-ATPase paused at three angles, each 120° apart, waiting for ATP to bind. Statistical analysis of the waiting time between the 120° steps indicated that each step was coupled with a single-ATP-hydrolysis reaction. Subsequently, this was confirmed directly by using a femtolitre chamber (Rondelez *et al*, 2005). The 120° step was further resolved into 90° and 30° substeps in a high-speed imaging experiment (Yasuda *et al*, 2001). The sizes of these substeps were later clarified to be 80° and 40° (Shimabukuro *et al*, 2003). Kinetic analyses of the substeps showed that the 80° substep is initiated by ATP binding and that the 40° substep is triggered by two consecutive reactions, each having a time

[1]Department of Mechanical Engineering, University of Tokyo, Tokyo 113-8656, Japan
[2]Institute of Scientific and Industrial Research, Osaka University, Mihogaoka 8-1, Ibaraki, Osaka 567-0047, Japan
[3]Florida State University, Tallahassee, Florida 32306-4370, USA
[4]Chemical Resources Laboratory, Tokyo Institute of Technology, Yokohama, Japan
[+]Corresponding author. Tel: +81 6 6879 8481; Fax: +81 6 6875 5724;
E-mail: hnoji@sanken.osaka-u.ac.jp

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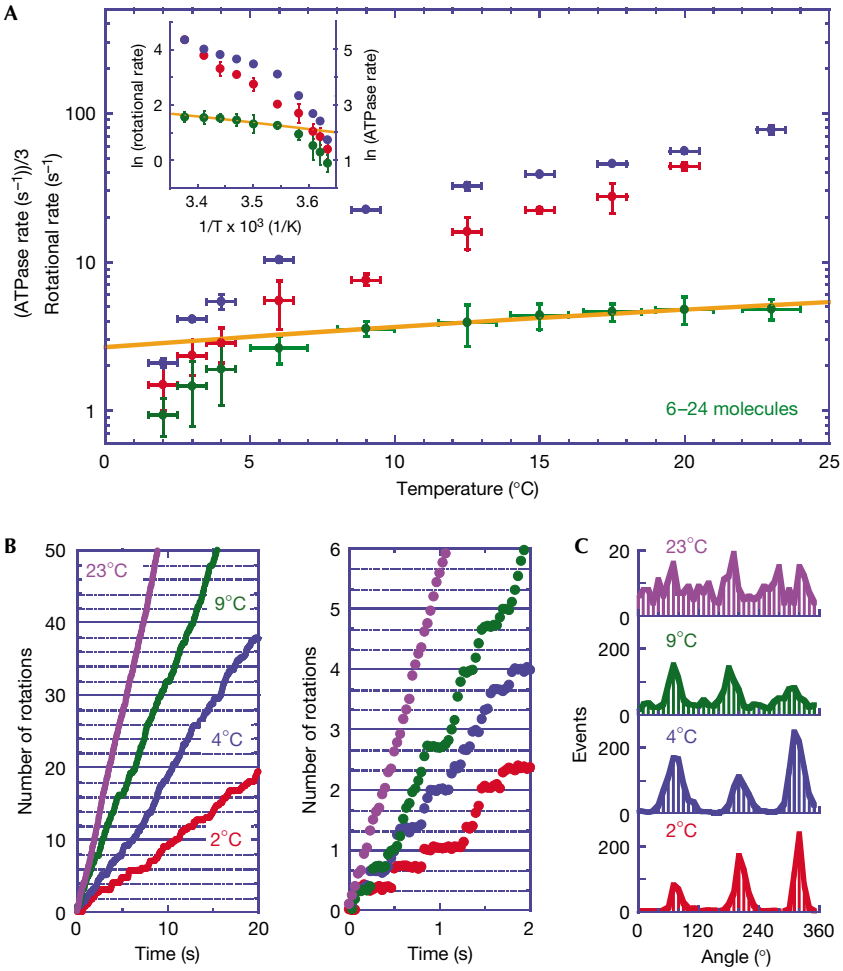

Fig 1 | Rotation of F_1-ATPase at low temperatures. (**A**) Temperature dependence of the rotational rate at 1 mM ATP. The rates determined in the rotational assay (green circles) and the rotational rates in bulk solution estimated as one-third of the ATP hydrolysis rate (red circles, the initial rate; blue circles, the maximum rate) were plotted; the inset shows the Arrhenius plot. Theoretical rotational rates (orange line) were calculated from the viscosity of water at each temperature assuming that the rotation against the viscous friction sets the pace of the overall reaction and that the torque of F_1-ATPase remained constant. (**B**) Time courses of rotations at 2 °C (red), 4 °C (blue), 9 °C (green) and 23 °C (purple) at 1 mM ATP (left), and magnified (right). (**C**) Histograms of angles from (**B**); binning interval for each angle was 10 °.

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, ATPγS (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 ($\phi = 0.2\,\mu m$) 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

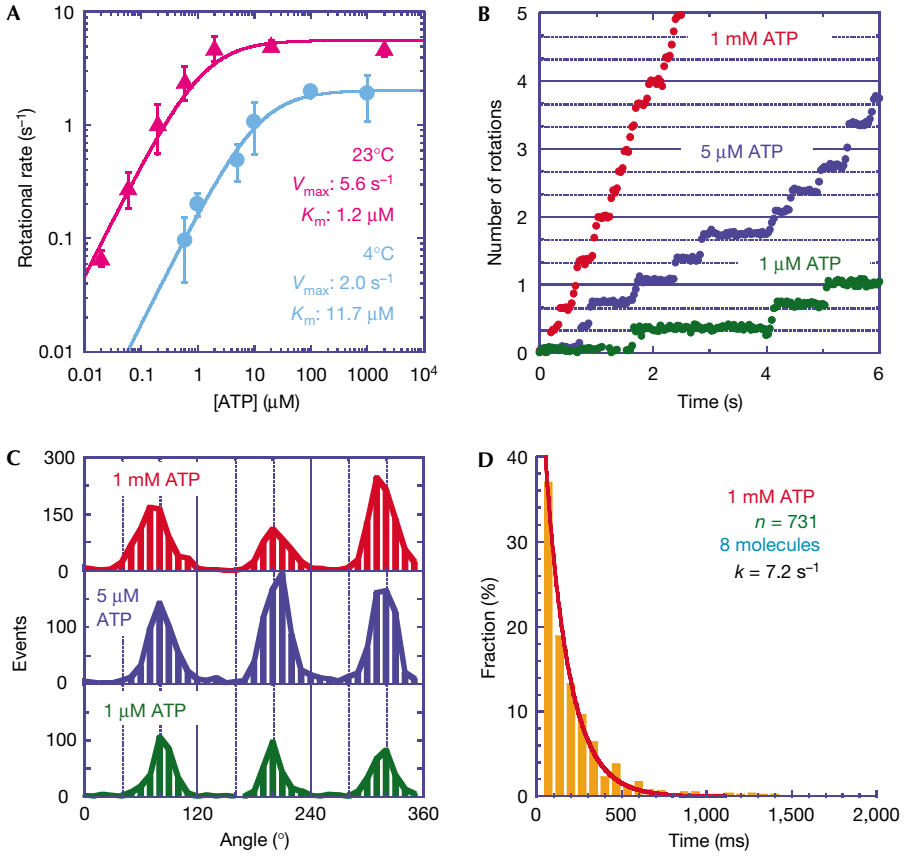

Fig 2 | Rotation at various ATP concentrations. (**A**) Michaelis–Menten curves determined at 23 °C (pink) and 4 °C (light blue). (**B**) Time courses of rotations at 4 °C and at 1 mM ATP (red), 5 μM ATP (blue) and 1 μM ATP (green). (**C**) Histograms of angles from (**B**). (**D**) Dwell-time histogram of the pause at 1 mM ATP. The rate constant of temperature-sensitive reaction was determined by fitting (red line); constant × exp(–*kt*).

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 F_1-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, Q_{10} 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 Q_{10} 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 formers 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 V_{max} and K_m values at 4 °C were 2.0 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 F_1-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 K_m, in which the time 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 ATPγS solution was replaced with an ATP solution during the rotation of an F_1-ATP molecule. We have shown that F_1-ATPase slowly hydrolyses ATPγS and transiently pauses at the catalytic angle until the execution of hydrolysis (Shimabukuro *et al*, 2003). In the presence of 1 mM ATPγS at 4 °C, F_1-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

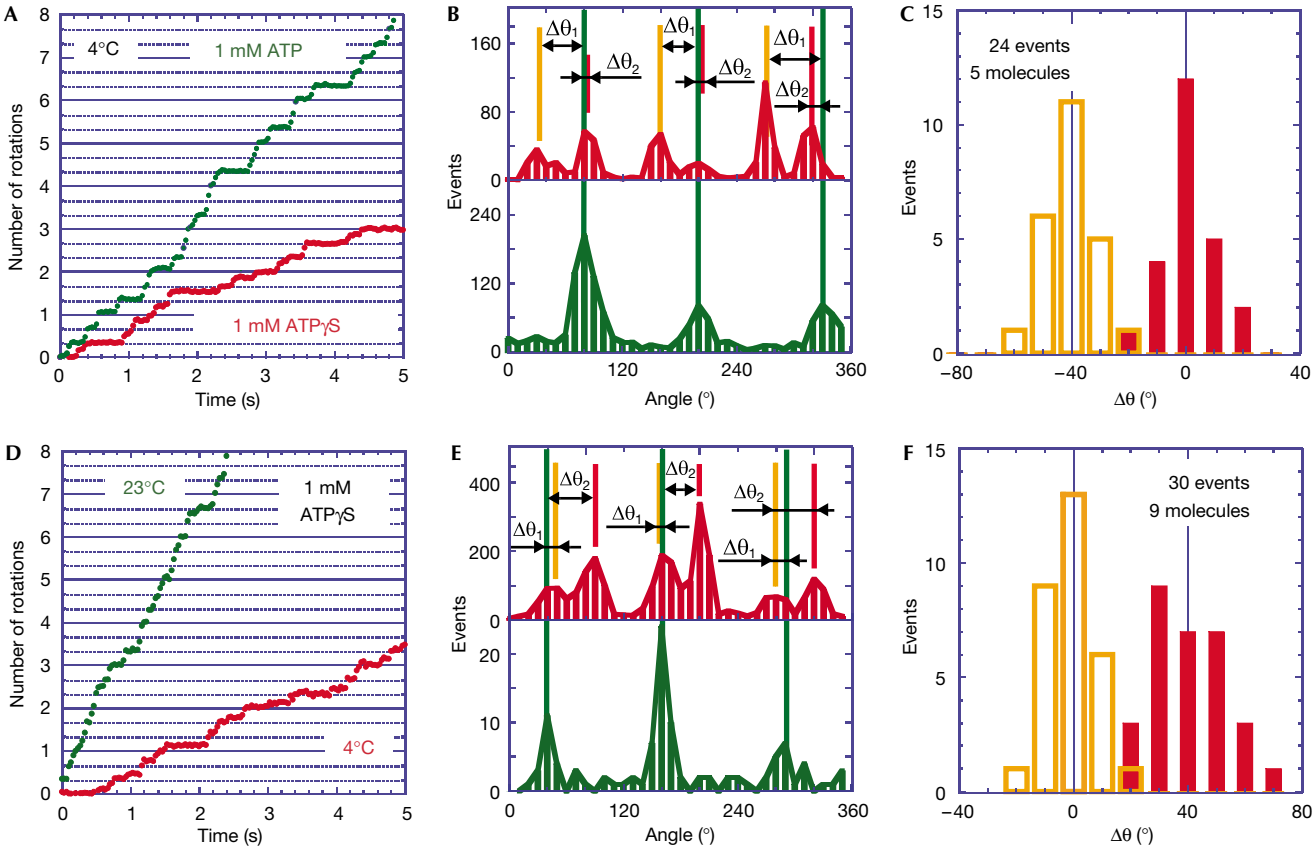

Fig 3 | Rotation driven by ATPγS. (**A**) Time courses of rotations of a single F_1-ATPase molecule driven by ATPγS (red) or ATP (green) at 4 °C. The 1 mM ATPγS buffer was replaced with the 1 mM ATP buffer. (**B**) Angle histograms of (**A**). (**C**) Histograms of the angular distances from the pause angle for the temperature-sensitive reaction (lower histogram) to that for ATPγS hydrolysis ($\Delta\theta_1$) or temperature-sensitive reaction ($\Delta\theta_2$) at 1 mM ATPγS (upper histogram). Orange and red bars represent the distributions of $\Delta\theta_1$ and $\Delta\theta_2$, respectively. (**D**) Time courses of rotations of a single F_1-ATPase molecule at 4 °C (red) or 23 °C (green) in the presence of ATPγS. Temperature was first set at 4 °C and increased to 23 °C. (**E**) Angle histograms of (**D**). (**F**) Histogram of the angular distances from the pause angle for ATPγS hydrolysis at 23 °C (lower histogram) to that for ATPγS hydrolysis ($\Delta\theta_1$) or temperature-sensitive reaction ($\Delta\theta_2$) at 4 °C (upper histogram). Orange and red bars represent the distributions of $\Delta\theta_1$ and $\Delta\theta_2$, respectively.

before the 40° or 80° substep. After replacement with 1 mM ATP solution, the pauses before the 40° substep disappeared, and the F_1-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 ($\Delta\theta_2$ in Fig 3B) was $+3.1 \pm 8.3$°, 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 ($\Delta\theta_1$) 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, F_1-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 ($\Delta\theta_1$ in Fig 3E), whereas that of the pause before the 80° substep from the ATPγS-hydrolysis angle ($\Delta\theta_2$ in Fig 3E) was $+42 \pm 12$° (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_i^{ADP} of 43 μM and $K_i^{P_i}$ of 1.8 mM. The K_i^{ADP} and $K_i^{P_i}$ were also determined in the bulk ATPase measurements to be 1.5 μM and 1.2 mM, respectively (supplementary Fig S3 online). Although the $K_i^{P_i}$ values were consistent between bulk and rotation assays, the K_i^{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_i^{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 P_i in solution. After observing the rotation at 1 mM ATP, a solution containing ADP or P_i 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 μM ATP also yielded similar results ($\Delta\theta$ in Fig 4H was -0.5 ± 7.6°). Thus, these results show that F_1-ATPase re-binds and releases ADP at the binding angle. Conversely, when 600 mM P_i 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 P_i, buffer-changing experiments were carried out at a lower ATP concentration of 10 μM. Fig 4F clearly shows that the pausing angle shifted from the binding angle in the presence of 600 mM P_i. Statistical analysis (Fig 4I) showed that $\Delta\theta$ was -29 ± 9.2° from the binding angle. Thus, it was clarified that P_i 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 P_i showed that P_i 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, P_i 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° (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°. Our finding that P_i 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 P_i dissociates once it binds to F_1-ATPase as ATP. With regard to this point, a single-molecule study using a chimaera 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, P_i 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 $6.6 \times 10^2\ s^{-1}$ from the Q_{10} factor. This rate is too fast to be detected even with high-speed imaging.

Al-Shawi *et al* (1997) reported a bulk ATPase measurement of *Escherichia coli* F_1-ATPase at temperatures ranging from 5 °C to 45 °C; however, strong temperature dependence was not found.

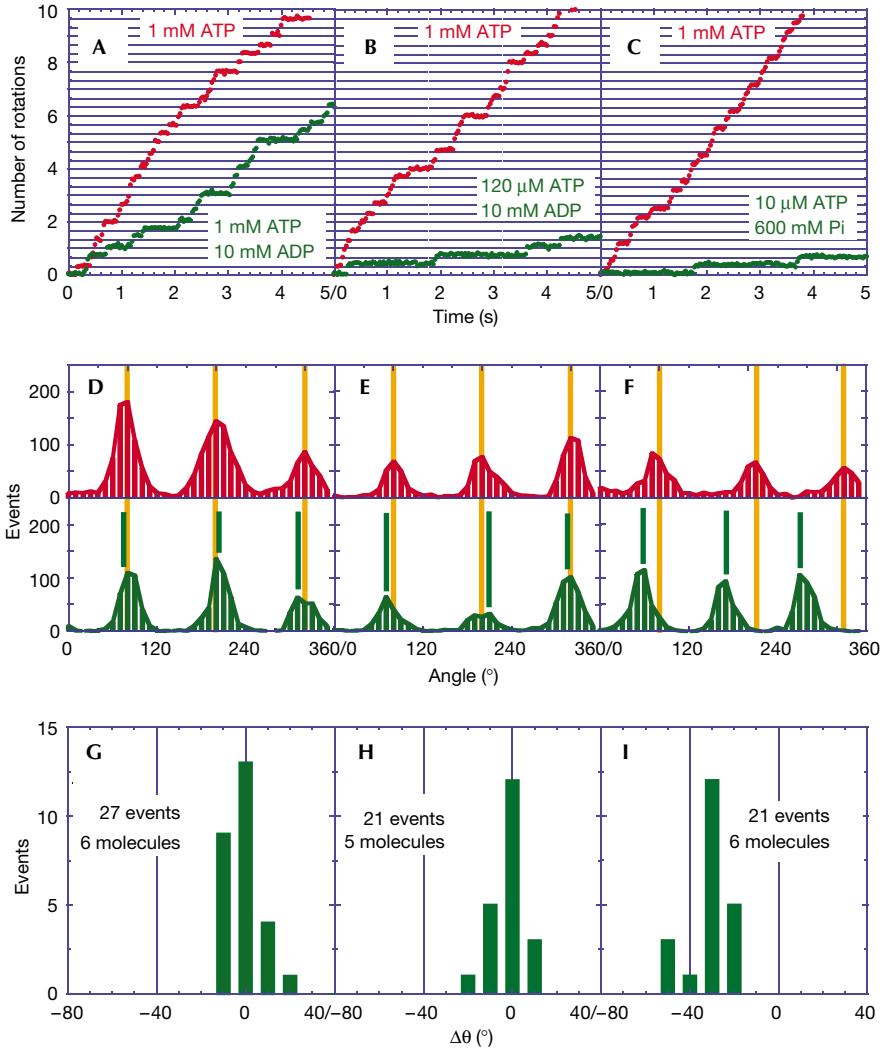

Fig 4 | Rotation in the presence of ADP or inorganic phosphate at 4 °C. (**A–C**) Time courses of rotations of a single F_1-ATPase molecule before and after buffer replacement. The 1 mM ATP buffer (red line) was replaced with (**A**) the 1 mM ATP + 10 mM ADP buffer (green line), (**B**) the 120 μM ATP + 10 mM ADP buffer, or (**C**) the 10 μM ATP + 600 mM P_i buffer. (**D–F**) Angle histograms of (**A**), (**B**) and (**C**), respectively. (**G–I**) Histograms of the angular distance from the pause at 1 mM ATP to the pause at (**G**) 1 mM ATP + 10 mM ADP, (**H**) 120 μM ATP + 10 mM ADP or (**I**) 10 μM ATP + 600 mM P_i.

In their study, the Q_{10} factor of ATPase of *E. coli* F_1-ATPase was estimated to be around 2 (refer to Fig 4; Al-Shawi *et al*, 1997). The ATPase of *E. coli* F_1-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 F_1-ATPase. Fig 1A also shows the Q_{10} factor of 2–4 from the ATPase rate at 9 °C to 23 °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* F_1-ATPase.

METHODS

Rotation assay. F_1-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 F_1-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 F_1-ATPase in solution was measured using an ATP-regenerating system, as reported previously (Rondelez *et al*, 2005). The initial hydrolysis rate (0–5 s^{-1} 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

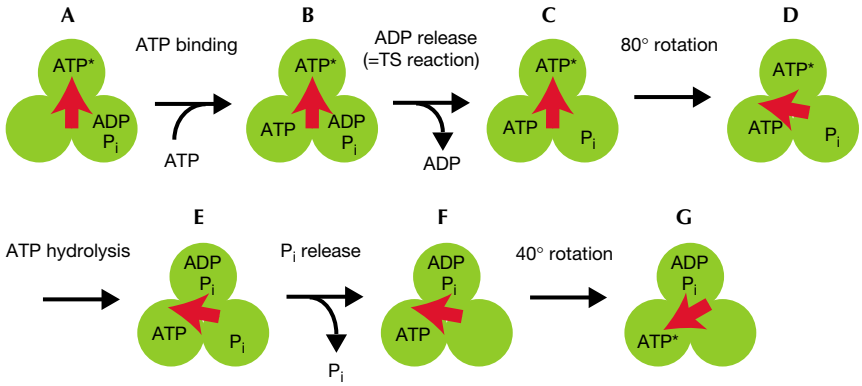

Fig 5 | Proposed scheme of chemical reaction and rotation of F_1-ATPase. Three green circles and a red arrow represent the chemical state of the three β-subunits and the γ-subunit, respectively. (**A**) represents the ATP waiting state, and the angular position of γ corresponds to the binding angle. ATP* represents ATP tightly bound to β-subunit but not yet hydrolysed. After new ATP binding to the empty β-subunit (**B**), ADP is released from the second β-subunit (**C**), followed by 80° rotation of the γ-subunit (**D**). Subsequently, ATP* in the third β-subunit is hydrolysed (**E**), and P_i is released (**F**), followed by 40° rotation of the γ-subunit (**G**). (**G**) is identical to (**A**).

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 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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