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Reversible control of F_1-ATPase rotational motion using a photochromic ATP analog at the single molecule level

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ABSTRACT

Motor enzymes such as F_1-ATPase and kinesin utilize energy from ATP for their motion. Molecular motions of these enzymes are critical to their catalytic mechanisms and were analyzed thoroughly using a single molecule observation technique. As a tool to analyze and control the ATP-driven motor enzyme motion, we recently synthesized a photoresponsive ATP analog with a *p-tert*-butylazobenzene tethered to the 2′ position of the ribose ring. Using *cis*/*trans* isomerization of the azobenzene moiety, we achieved a successful reversible photochromic control over a kinesin-microtubule system in an *in vitro* motility assay. Here we succeeded to control the hydrolytic activity and rotation of the rotary motor enzyme, F_1-ATPase, using this photosensitive ATP analog. Subsequent single molecule observations indicated a unique pause occurring at the ATP binding angle position in the presence of *cis* form of the analog.

1. Introduction

ATP is regarded as the energy currency in biological systems, and is used for various cellular activities. These activities are primarily performed by ATPases, which hydrolyze ATP into ADP and Pi, and harness the energy to drive various chemical reactions in the cell.

Most cellular ATP is synthesized by ATP synthase, which is ubiquitous in bacteria, plants, and animals. ATP synthase, also known as F_oF_1, produces ATP from ADP and Pi using electrochemical proton gradient across energy producing membranes [1]. The enzyme comprises a membrane embedded part F_o, which works as proton pump, and a water soluble part F_1, which contains catalytic sites for ATP synthesis and hydrolysis. Isolated F_1 solely catalyzes ATP hydrolysis using a rotary catalysis mechanism. Therefore, F_1 is known as F_1-ATPase and the minimum catalytic core of F_1 is known to comprise $\alpha_3\beta_3\gamma$ [2]. In 1997, Noji et al. directly visualized rotation of the γ subunit in the $\alpha_3\beta_3$ hexagon under an optical microscope by attaching a fluorescent-labeled actin filament to the γ subunit of the $\alpha_3\beta_3\gamma$ complex, which was fixed on a glass surface [3]. Following this sophisticated experiment, a series of detailed molecular studies elucidated the nature of the rotation at the atomic scale, and finally, the coupling scheme, including γ rotation and chemical reactions (hydrolysis and product release), was described [4–6].

Till date, the biochemistry of ATPase has been studied using various synthetic ATP analogs. For example, the fluorescent ATP analog 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) [7] was used to investigate the nucleotide binding sites on ATPase, and the interaction with ATPase was directly detected as changes in absorption or fluorescence of the analog [8]. Nonhydrolyzable analog, adenylyl imidodiphosphate (AMP-PNP) [9], was used to crystallize ATPase for X-ray structure determination [10]. In addition, caged ATP [11], in which the terminal phosphate is esterified with a blocking group, releases free ATP by UV irradiation, enabling precise control of reaction initiation.

Abbreviations: F_oF_1, F_oF_1-ATP synthase; F_1 (F_1-ATPase), catalytic moiety of ATP synthase; F_o, membrane sector of ATP synthase; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; AMP-PNP, adenylyl imidodiphosphate; VIS light, visible light; ATP-AzotBu, ATP analog containing azobenzene at the 2′ position of the ribose and a *tert*-butyl group at the azobenzene terminus; *trans*-ATP-AzotBu, *trans* form of ATP-AzotBu; *cis*-ATP-AzotBu, *cis* form of ATP-AzotBu; PSS, photostationary state.

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Application of light controllable analogs to this motor enzyme may facilitate future study on the development of bio-devices. Thus, we recently synthesized a novel photochromic ATP analog containing azobenzene at the 2′ position of the ribose and a *tert*-butyl group at the azobenzene terminus [12] (hereafter referred as ATP-AzotBu). The most remarkable feature of the analog is to exhibit reversible conformational changes of azobezene upon light irradiation of particular wavelengths (Fig. S1). UV light irradiation promotes *trans*-to-*cis* isomerization of ATP-AzotBu, forming a *cis*-rich photostationary state (PSS). Following irradiation with visible (VIS) light promotes *cis*-to-*trans* isomerization of ATP-AoztBu, which reaches a *trans*-rich PSS. Azobenzene derivatives have been applied as photocontrollable modulators of various biomolecules such as peptides, ligands, and oligonucleotides [13]. In fact, ATP-AzotBu successfully controlled the motility of the linear motor enzyme, kinesin, along with isomerization [12]. In this study, we applied ATP-AzotBu as a substrate for the rotary motor enzyme, F_1-ATPase. Using spectroscopic measurements and single molecule observation technique, we demonstrated the reversible photochromic control of activity of F_1-ATPase.

2. Materials and methods

2.1. Materials

ATP-AzotBu was synthesized as previously described [12].

2.2. Protein

A mutant F_1-ATPase ($\alpha_3\beta_3\gamma$ complex) of the thermophilic cyanobacteria *Thermosynechococcus elongatus* BP-1 was used in this study. In the mutant complex, an insert sequence of γ was deleted to effectively release F_1-ATPase from ADP inhibition [14]. Several mutations for single molecule analysis were introduced as well [15]. The plasmid preparation and the complex purification were described in the Supplemental data.

2.3. Spectrophotometric measurements of ATP and ATP-AzotBu hydrolysis activity

Hydrolysis of ATP and ATP-AzotBu was measured using an ATP regenerating system as previously described [16]. The assay mixture contained 50 mM HEPES/KOH (pH 8.0), 100 mM KCl, 0.5 mM $MgCl_2$, 100 μM ATP or ATP-AzotBu, 6.8–11 units/ml pyruvate kinase, 4.5–7.5 units/ml lactate dehydrogenase, 2 mM phosphoenolpyruvate, and 0.4 mM NADH. The assay was performed at 25 °C. For ATP-AzotBu isomerization, a UV light illuminator (UV-400; Keyence, Osaka, Japan) was used to promote *cis* isomerization, and a VIS light illuminator (LC-700SV; Dynatec, Tokyo, Japan) was used to promote *trans* isomerization. UV and VIS light irradiations were performed for 15 and 30 s, respectively, with their maximum outputs at PSS.

2.4. Single molecule analyses with ATP or ATP-AzotBu

Rotation assays were performed as previously described [16]. The assay buffer contained 50 mM HEPES/KOH (pH 8.0), 100 mM KCl, 0.5 mM $MgCl_2$, 20 units/ml pyruvate kinase, 2 mM phosphoenolpyruvate, and various concentrations of ATP or ATP-AzotBu. The assay was performed at 25 °C. Isomerization of ATP-AzotBu was achieved using the above mentioned irradiation devices. UV and VIS light irradiation was performed for 25 and 90 s, respectively, with maximum output. We placed a sharp cut filter into the optical system of microscope to absorb light at ⩽600 nm to prevent unexpected isomerization by the light source for the microscope.

3. Results

3.1. Light-triggered reversible control of F_1-ATPase activity using ATP-AzotBu

ADP inhibition is a typical strong inhibition mode for F_1-ATPase and it may make the interpretation of the experimental results complicated. Therefore we applied the mutant cyanobacterial $\alpha_3\beta_3\gamma$ complex which is less prone to ADP inhibition in this study.

First, we examined *trans* form ATP-AzotBu (*trans*-ATP-AzotBu), as a substrate, and found that the compounds successfully triggered hydrolysis by F_1-ATPase at a rate of 3.1 ± 0.3 s^{-1} (Fig. 1C, left bar). In contrast, ATP was hydrolyzed at a rate of 75 ± 4 s^{-1} under the same experimental condition (Fig. S3B). Although the basal activity in the presence of *trans*-ATP-AzotBu was only 4% of that in the presence of ATP, ATP-AzotBu acted as a substrate for F_1-ATPase.

Second, we performed the measurements of ATP-AzotBu with *cis*-rich PSS, which was prepared by UV light-irradiation. By ^{1}H NMR analyses, it was revealed that the azobenzene compounds in *cis*-rich PSS contains 92% of *cis* form and 8% of *trans* form [12]. We found that *cis*-rich ATP-AzotBu triggered the hydrolysis reaction at a rate of 0.41 ± 0.15 s^{-1} (Fig. 1D, left bar), which was 13% of that by *trans*-ATP-AzotBu hydrolysis rate. In this coupling assay system, the pyruvate kinase catalyzed reaction was not the rate limiting step of the ATP regenerating system, even when ATP-AzotBu was used as a substrate, because the apparent activities exhibited linear dependency on the concentration of F_1-ATPase used in the assay.

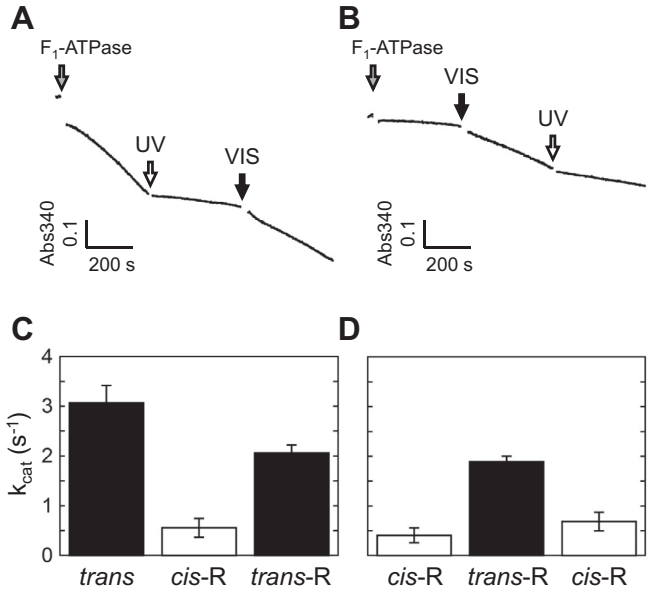

Fig. 1. ATP-AzotBu hydrolysis activity of F_1-ATPase. (A, B) Hydrolysis activity was measured in the presence of 100 μM ATP-AzotBu using ATP regenerating system. Time courses of the measurement initiating from *trans* form (A) and *cis*-rich form (B) are presented. After addition of the enzyme (gray arrows), UV (open arrows), and VIS (closed arrows) light irradiation promoted isomerization of ATP-AzotBu at the indicated times. Because *trans* and *cis*-rich forms of ATP-AzotBu have different absorbances at 340 nm (Fig. S1), absorbance values were modified to clarify slope differences. Raw data is presented in Fig. S2. (C, D) ATP-AzotBu hydrolysis in each azobenzene form was determined from steady state slopes of the corresponding time courses (A, B). Closed and open bars represent activities in the presence of *trans*, *trans*-rich (*trans*-R), and *cis*-rich (*cis*-R) forms, respectively. Three independent experiments were averaged. Error bars represent SD.

To investigate changes in hydrolysis rates by photo-conversion and the reversibility of the changes in these rates, we promoted isomerization of ATP-AzotBu by UV or VIS irradiation in sequential measurements. Before the measurement, we confirmed that UV or VIS light irradiation used for isomerization revealed no effect on the ATP hydrolysis activity (Fig. S3). Fig. 1A and B present time courses of ATP-AzotBu hydrolysis upon isomerization of the substrates by UV or VIS light irradiation. The measurement of hydrolysis of *trans*-ATP-AzotBu was initiated after addition of the enzyme (gray arrow) (Fig. 1A). UV light was then irradiated to convert the azobenzene moiety to *cis*-rich form (open arrow), resulting in a remarkable decrease in enzyme activity. Subsequently, VIS light was applied again to convert the azobenzene moiety to the *trans*-rich form (closed arrow), leading to the recovery of enzyme activity. These data indicate that the hydrolysis of ATP-AzotBu by F_1-ATPase can be reversibly controlled by photochromic isomerization. Enzyme activities in the presence of each substrate form were determined from the corresponding slopes at the steady states (Fig. 1C), which indicate that hydrolysis activity in the presence of *cis*-rich ATP-AzotBu was 18% of that in the presence of *trans* form. When ATP-AzotBu was converted again from *cis*-rich to *trans*-rich form by VIS light irradiation, enzyme activity was recovered to 67%. We also measured the activity initiated from *cis*-rich ATP-AzotBu obtained in Fig. 1B and confirmed the reversibility by isomerization (Fig. 1D).

3.2. Single molecule observations of F_1-ATPase rotation in the presence of ATP-AzotBu

In order to evaluate the influence of photo-isomerization of azobenzene moiety on the catalytic steps, single molecule observation technique was applied. We successfully observed rotation of the γ subunit in the presence of ATP-AzotBu (Fig. 2). We then examined the reversibility of rotation rates by light-triggered isomerization of ATP-AzotBu at the single molecule level. Fig. 2A and B present typical time courses of rotations initiated from *trans*-ATP-AzotBu and *cis*-rich ATP-AzotBu, respectively. After observation of rotation for several minutes, UV (open arrow) or VIS (closed arrow) light was directly irradiated to the whole glass chamber on the microscope stage to promote isomerization of ATP-AzotBu in the buffer solution. Subsequent irradiation with VIS or UV light reconverted the substrate to the form at the initial phase of the experiment. Time courses presented in Fig. 2A indicated that the revolutions were decelerated upon UV light irradiation, and then accelerated upon VIS light irradiation. In case of the observation obtained in presence of *cis*-rich ATP-AzotBu (Fig. 2B), the revolutions were accelerated upon VIS light irradiation, and then decelerated upon UV light irradiation. To evaluate these data quantitatively, we calculated the rotation rates of each single molecule by dividing total revolutions with the corresponding observation time period of each azobenzene form. The averaged rotation rates were then

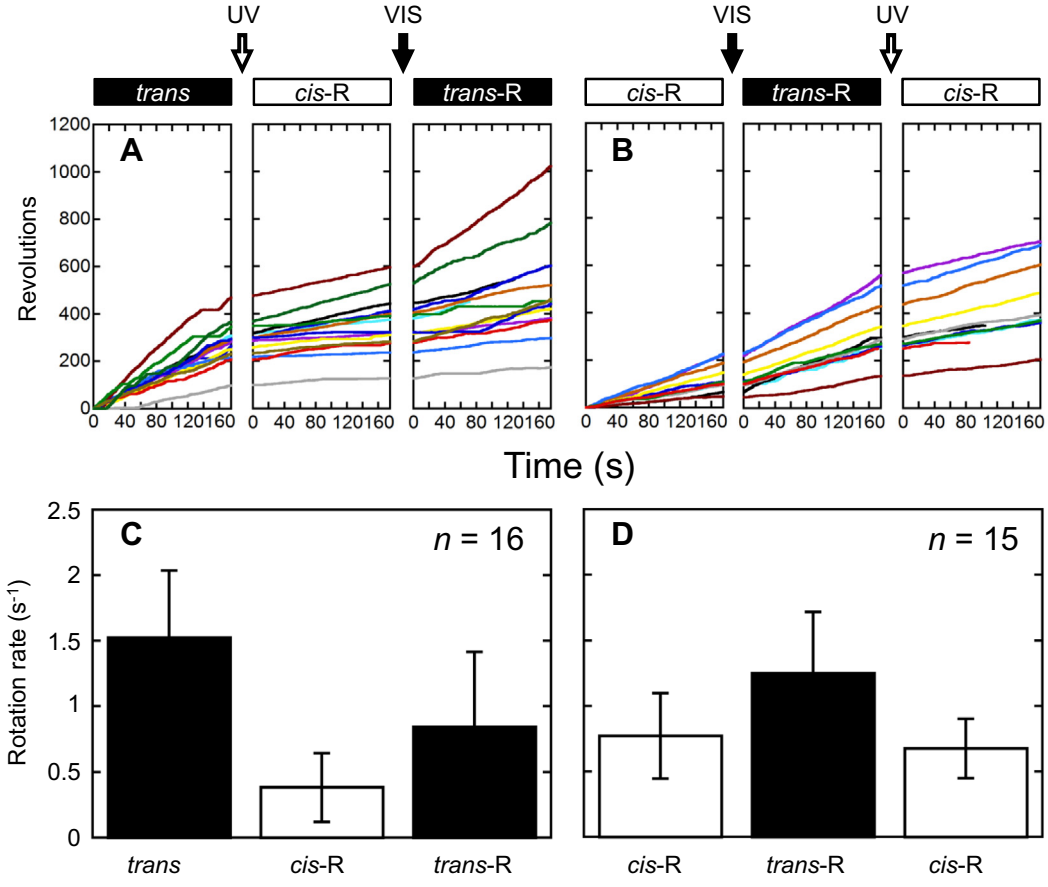

Fig. 2. Single molecule analysis of F_1-ATPase with ATP-AzotBu. (A, B) Typical time courses of the rotation in the presence of 100 μM ATP-AzotBu initiating from *trans* form (A) and initiating from *cis*-rich form (B). During the observation, UV (open arrows) and VIS (closed arrows) light irradiation promoted isomerization of ATP-AzotBu, as indicated in the upper boxes; "*cis*-R" and "*trans*-R" represent *cis*-rich and *trans*-rich, respectively. (C, D) Rotation rates in the presence of each azobenzene form phase were calculated from total revolutions per observation time period in the corresponding time courses (A, B). Closed and open bars represent rotation rates in the presence of *trans*, *trans*-rich, and *cis*-rich forms, respectively. Error bars represent SD.

calculated (Fig. 2C and D). In case of the observation obtained in presence of *trans* form (Fig. 2C), rotation rates were decreased to 25% after irradiation with UV light, and were then recovered to 56% of the initial level after VIS light irradiation. In contrast, rotation rates in the presence of *cis*-rich ATP-AzotBu were increased to 161% after irradiation with VIS light (Fig. 2D). Subsequent irradiation with UV light, rotation rate was restored almost the same level as that of the initial phase of the experiment. In agreement with spectroscopic measurements, these data clearly indicate reversible control of F_1-ATPase rotation by isomerization of substrate ATP-AzotBu at the single molecule level.

3.3. Specific pauses in the presence of cis-rich ATP-AzotBu

In the presence of 100 μM *cis*-rich ATP-AzotBu, we found that rotation exhibited three discrete pauses that were separated by 120° (hereafter referred as *cis*-rich form pause; Fig. 3B). This stepping rotation was not clear in the presence of 100 μM of *trans*-ATP-AzotBu. Two distinct stable conformations during the catalytic cycles were known for F_1-ATPase [17]. When the ATP binding position is assigned as 0°, ATP hydrolysis occurs at the 80° position of rotated γ [18]. Therefore, we attempted to determine the position of the *cis*-rich form pause by solution replacement method (Fig. 3). In these experiments, we initiated observations with *cis*-rich ATP-AzotBu, and then replaced the reaction solution with buffer containing 200 nM ATP. At very low ATP concentrations, ATP binding becomes rate limiting and three distinct steps during rotation can be observed [19]. These three discrete pauses were attributed to ATP binding positions. Fig. 3A presents the results of rotation during solution replace experiment. After several minutes of rotation in the presence of 200 nM ATP, the reaction solution was replaced again with buffer containing 1 mM ATP. The rotation rates were greatly increased (Fig. 3A, black lines) and stepping rotations were completely obliterated (Fig. 3B, black lines), indicating that the stepping rotations in the presence of *cis*-rich ATP-AzotBu and 200 nM ATP were not generated by artificial rotation barriers. We concluded that the *cis*-rich form pause occurs at the same position of the ATP binding dwell (Fig. 3C) because the angle deviance ($\Delta\theta$) from the ATP binding pause to *cis*-rich form pause was only 1.8 ± 15.5° (30 events for 10 molecules).

3.4. Substrate dependence of rotation behavior

To determine the kinetic parameters of F_1-ATPase with ATP-AzotBu, we performed single molecule analyses of F_1-ATPase rotation in the presence of various concentrations of ATP and ATP-AzotBu (Fig. 4). V_{max} and K_m values were then determined by fitting the plots with Michaelis–Menten kinetics. V_{max} values in the presence of ATP, *trans*-ATP-AzotBu, and *cis*-rich ATP-AzotBu were 5.5, 2.4, and 0.88 s^{-1}, respectively (Table 1). In the single molecule experiment, large beads were attached to the complex. Thus, friction due to viscosity of water decreased the motion rate of γ and strongly affected the V_{max} values especially in the presence of ATP. In contrast, the motion rate of γ did not become a rate limiting step in the presence of ATP-AzotBu. Thus, the difference between V_{max} values obtained from single molecule analysis in the presence of ATP and *trans*-ATP-AzotBu (5.5 s^{-1} vs. 2.4 s^{-1}) was smaller than that between V_{max} values obtained from spectroscopic measurement (75 s^{-1} vs. 3.1 s^{-1}). However, rotation rates (V_{max} value) in the presence of *trans*-ATP-AzotBu were significantly higher than that in the presence of *cis*-rich ATP-AzotBu, which is mostly consistent with the results obtained from spectroscopic activity measurements. In contrast, K_m values of ATP, *trans*-ATP-AzotBu, and *cis*-rich ATP-AzotBu were not significantly different (Table 1).

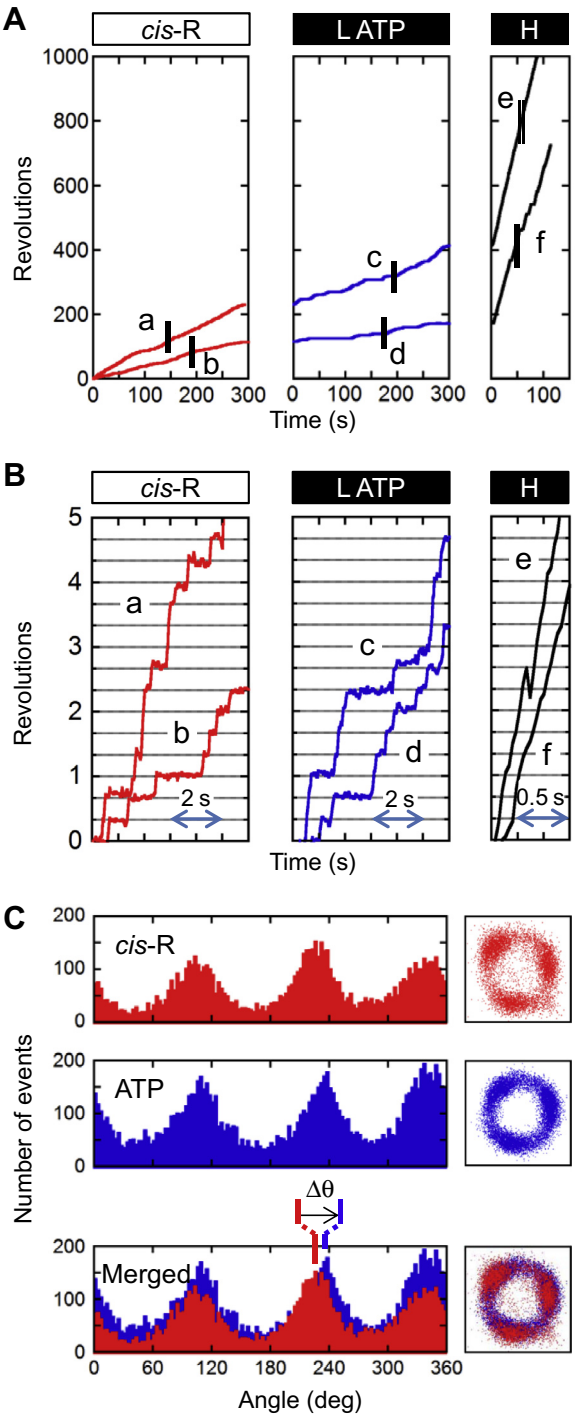

Fig. 3. Stop angular positions of rotations in the presence of *cis*-rich ATP-AzotBu. (A) Time courses of solution replacements are presented. After observing rotation for 5 min in the presence of 100 μM *cis*-rich ATP-AzotBu (red lines), the reaction mixture was replaced with a solution containing 200 nM ATP (blue lines). After 5 min observation with 200 nM ATP, the reaction mixture was again replaced with a solution containing 1 mM ATP (black lines). Solution conditions are indicated in the upper boxes ("L ATP" and "H" indicate 200 nM and 1 mM ATP, respectively). (B) Magnified time courses of each solution are labeled as a–f in (A) and are shown using the color codes of the traces. (C) Histograms of angular distributions of beads with 100 μM *cis*-rich ATP-AzotBu (upper) and 200 nM ATP (middle) are presented. The bottom figure represents a merged histogram. The right panels represent the traces of bead centroids corresponding with the histograms on the left. 10 different molecules were observed. $\Delta\theta$ represents the angle distance from the ATP binding pause to *cis*-rich form pause. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

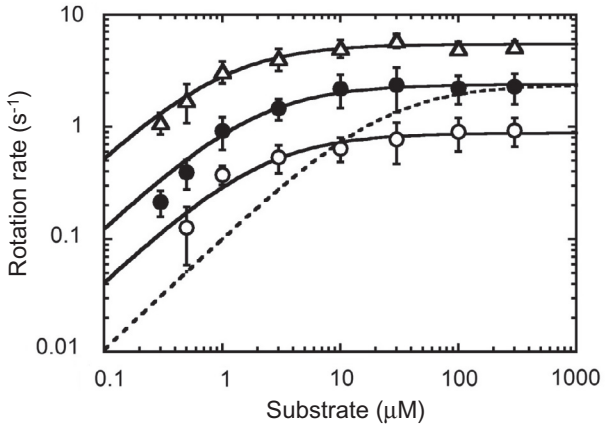

Fig. 4. Rotation at various ATP or ATP-AzotBu concentrations. Rotation rates in the presence of ATP (open triangles), *trans* (closed circles), and *cis*-rich (open circles) forms of ATP-AzotBu were determined in the presence of various substrate concentrations. Each rate was determined from continuous rotations for 10–20 s. Error bars represent SD (n = 4–11). The plots were fitted with Michaelis–Menten kinetics (solid lines). The theoretical rotation rates for residual *trans*-ATP-AzotBu (8% content) after UV irradiation are indicated as a dotted line. In the hypothesis, *cis*-ATP-AzotBu does not act as a substrate.

Table 1
V_{max} and K_m values of F_1-ATPase in the presence of each substrate. V_{max} and K_m values were determined by fitting the plots with Michaelis–Menten kinetics (Fig. 4).

	V_{max} (s^{-1})	K_m (μM)
ATP	5.5	0.94
ATP-AzotBu *trans* form	2.4	1.8
ATP-AzotBu *cis*-rich form	0.88	2.0

4. Discussion

In this study, we used the photochromic ATP analog, ATP-AzotBu [12], to modulate rotary molecular motor activity of F_1-ATPase. ATP-AzotBu successfully triggered and controlled ATP hydrolysis by F_1-ATPase (Fig. 1) and rotation at the single molecule level (Fig. 2) by isomerization following photo irradiation.

ATP-AzotBu *cis*-rich form triggered reduced hydrolysis activity and rotation rate of F_1-ATPase (18% and 25% to those by *trans* form, respectively) (Figs. 1 and 2). Moreover, rotation in the presence of *cis*-rich ATP-AzotBu revealed three stepping rotations (Fig. 3), whereas that in the presence of *trans*-ATP-AzotBu was not interrupted. In Fig. 3C, we determined that the *cis*-rich form pause occurs at the same position as ATP binding. A possible explanation for the observed pause is that *cis*-rich ATP-AzotBu does not act as a substrate, and the remaining *trans* form (8 μM of the 100 μM ATP-AzotBu) exhibits the stepping rotations because the amount of remaining *trans* form are quite low. However, we could not observe clear stepping rotations in the presence of 3 or 10 μM *trans*-ATP-AzotBu (Fig. S4B). To investigate this discrepancy, we plotted theoretical kinetics curves (Fig. 4, dotted line) calculated from the remaining 8% *trans* form, but found a poor fit with the experimental data obtained in the presence of *cis*-rich form (open circles). Thus, the observed pauses were difficult to attribute to remaining *trans*-ATP-AzotBu after UV light irradiation, and should be attributed to *cis* form.

At low substrate conditions (<10 μM), rotation rates were higher than theoretical rates (Fig. 4), suggesting that *cis*-ATP-AzotBu also acts as a substrate for F_1-ATPase, although the hydrolysis rate was quite slow. The stepping rotations exhibited by *cis*-ATP-AzotBu may be explained if the binding of *cis*-ATP-AzotBu is slower than that of *trans* form. However, ADP release and ATP binding reportedly occurs at the same angular position [4,5]. Thus, one of the possibilities is that the release of *cis*-ADP-AzotBu is severely delayed. In fact, several reports of ATP analogs comprising aromatic moieties at the ribose position of adenosine, such as TNP-ATP [8] and 3′-*O*-(4-benzoyl) benzoyl ADP [20], strongly affect the affinity to the enzyme. To provide the definitive conclusion on the difference in F_1-ATPase activity and rotation in the presence of *cis* and *trans* forms of ATP-AzotBu, structural information regarding the entry of the analog into the catalytic pocket is required.

In kinesin motility assays, sliding velocities in the presence of 100 μM *cis* and *trans* forms of ATP-AzotBu were 19% and 30% of that in the presence of 100 μM ATP, respectively [12]. In contrast, the hydrolytic activity of F_1-ATPase in the presence of 100 μM ATP-AzotBu *trans* and *cis*-rich form was only 4.1% and 0.55% of that in the presence of 100 μM ATP, respectively (Fig. 1 and Fig. S3). Thus, the kinesin-microtubule system was more tolerant of varying substrates than F_1-ATPase. Although structural studies of F_1-ATPase or kinesin bound ATP-AzotBu have not been performed, interpretations can be ascertained in comparisons with ADP (or ATP) bound structures (Fig. S5). Nucleotide binding sites of F_1-ATPase are located at the interface of the α and β subunits, and are completely covered by surrounding residues (Fig. S5A and B). In contrast, the nucleotide binding site of the kinesin motor domain appears to be more open (Fig. S5C and D). Hence, the kinesin nucleotide binding pocket may have a weaker interaction with the adenosine moiety of ATP than that of F_1-ATPase. This assumption is supported by comparisons of K_m values for ATP with F_1-ATPase [0.94 μM from Table 1 and 15 μM from [17]] and with kinesin motors [44 μM [21], 85 μM [12]]. Thus, kinesin may bind ATP-AzotBu more easily at the nucleotide binding sites even though the substrate is modified at its ribose position. Moreover, substrate specificity of F_1-ATPase for ATP may be higher than that of kinesin, resulting in remarkable decrease in the activity of F_1-ATPase with ATP-AzotBu. In contrast with F_1-ATPase, *cis*-rich ATP-AzotBu triggers higher velocity of kinesin motors in comparison with *trans*-ATP-AzotBu [12]. This difference in the response to isomerization may be attributed to the difference in the interactions between the adenosine moiety and surrounding resides of the nucleotide binding pocket.

In the present study, we revealed that ATP-AzotBu acts as a substrate for F_1-ATPase, strongly suggesting that ATP-AzotBu is synthesized from ADP-AzotBu and Pi by ATP synthases. In addition, the pyruvate kinase used for the ATP regenerating system was applicable for ATP-AzotBu regeneration. Thus, this ATP analog may be applied to control ATP-related enzymes *in vivo*. We therefore anticipate that azobenzene based nucleotide analog is a promising molecular tool for developing bio-devices. Further analyses of synthetic azobenzene compounds using single molecule techniques and structural methods will lead us to prepare complete on/off switches for stringent artificial regulation of ATP-related enzymes. In the biochemical research aspects, azobenzene based nucleotide analog will be useful for researching substrate specificities for the various nucleotide-relating enzyme such as G-protein and ABC transporter.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.117.

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