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A photochromic ATP analogue driving a motor protein with reversible light-controlled motility: controlling velocity and binding manner of a kinesin–microtubule system in an *in vitro* motility assay†

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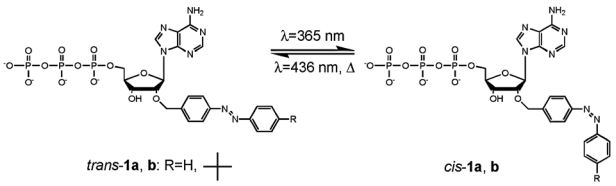
We synthesized two photochromic ATP analogues (ATP-Azos) featuring azobenzene derivatives tethered at the 2′ position of the ribose ring. In the presence of the ATP-Azo tethering *p-tert*-butylazobenzene, we observed reversible photo-control of the motility, velocity and binding manner, of a kinesin–microtubule system in an *in vitro* motility assay.

Adenosine 5′-triphosphate (ATP) is a biogenic molecule that functions as an energy donor and interacts with various proteins, ATPases. To investigate and artificially control the functions of ATPases, several photoresponsive ATP analogues, such as fluorescent-labeled ATPs[1] and caged-ATPs,[2] have been developed. The caged-ATP system is unique in that the hydrolysis of ATP mediated by an ATPase is initiated by UV light irradiation to cleave the *o*-nitrophenyl protecting group of the γ-phosphate unit of the ATP derivative. This photo-cleavage process is irreversible, therefore it is impossible to modulate the hydrolysis rate with desired timing once the protecting group has been released. An ATP analogue having the potential to reversibly photo-control the functions of ATPases would be a promising molecule to control various biochemical phenomena. In this regard, an ATP analogue possessing a photochromic unit that could undergo reversible conformational changes would be a candidate molecule for the reversible control of ATPase activity under irradiation with light. Azobenzene, which exhibits reversible conformational changes upon irradiation with light of various wavelengths, is often applied as a photo-controllable mediator of the functions of proteins[3] and synthetic molecules.[4–8] The photo-control of protein functions has been studied using azobenzene derivatives conjugated to peptides or proteins. However, there have been few studies of the development of small-ligand nucleotides connected to azobenzene derivatives.[9]

In this study, we synthesized azobenzene derivatives of ATP, ATP-Azos, as a novel class of ATP analogues. Herein, we demonstrate the reversible photo-control of a motile property of kinesin, a kind of ATPase motor protein, through an *in vitro* motility assay performed by utilizing an ATP-Azo.



Scheme 1 Photoresponsive ATP analogues **1a** and **1b**. Reversible *trans* (left)/*cis* (right) isomerizations of the azobenzene derivative moieties.

We designed our ATP analogues such that a single azobenzene moiety would be appended to the ribose ring of the ATP unit, in consideration of the fact that various ATP analogues modified at these positions can still interact with ATPases.[1,10] We synthesized ATP analogues tethering an azobenzene or *p-tert*-butylazobenzene moiety at the 2′ position of the ribose ring (Scheme 1) using the approach shown in Schemes S1 and S2 (see "Syntheses of Compounds", ESI†).[11] This synthetic pathway allows us to modify ATP only at the 2′ position of the ribose ring, thereby avoiding any potential confusion, due to the presence of 2′ and 3′ isomers, when interpreting the experimental results[12] and eliminating any possible contamination of ATP, in contrast to other syntheses starting from ATP. The resultant ATP-Azos, **1a** and **1b**, were obtained as their sodium salts.

We examined the photoisomerization behavior of ATP-Azos through alternating irradiation with UV light (λ = 365 nm), inducing *trans*-to-*cis* isomerizations of the azobenzene moieties of the ATP-Azos, and visible light (λ = 436 nm), inducing their reverse *cis*-to-*trans* isomerizations [Scheme 1; Fig. S1 and S2 (presenting the examples for **1b**), ESI†].

We used an *in vitro* motility assay of kinesin[13] to assess the properties of the ATP-Azos in their different isomeric states as substrates of ATPase, thereby allowing the ATPase activity to be microscopically visualized in terms of dynamic movements. In the assay, we confirmed that fluorescent-labeled microtubules (MTs)[14] were driven by recombinant kinesin (kinesin-1) molecules coated on the glass surface in the presence of ATP-Azos added instead of ATP, and evaluated the motility behaviors of the MTs (see "Observation of Motility of Kinesin–Microtubule System", Fig. S3, ESI†).

Table 1 and Fig. 1 display the sliding velocities of the MTs driven by the kinesins at various concentrations of the ATP-Azos

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Table 1 Sliding velocity of MTs in the presence of each substrate

	Sliding velocity of MTs				
	Average ± s.e.m. (nm s^{-1}), n = 15–50				
Concentration	*trans*-**1a**	*cis*-**1a**	*trans*-**1b**	*cis*-**1b**	ATP
1 mM	372 ± 7	383 ± 7	nd	236 ± 14	656 ± 26
100 μM	179 ± 4	175 ± 3	75 ± 3	117 ± 3	~390[a]

[a] Estimation (see Fig. S4, ESI).

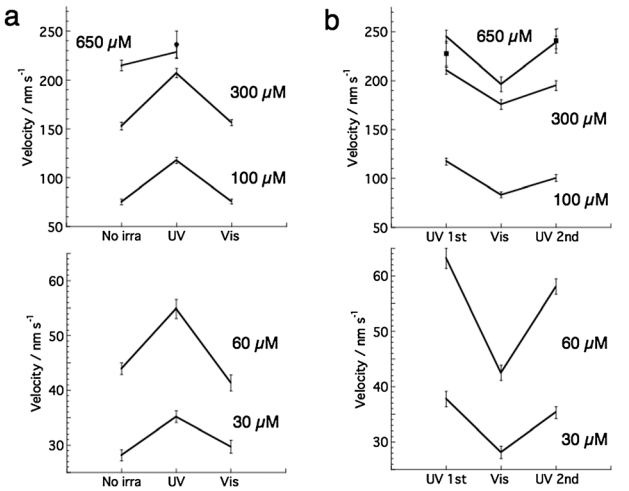

Fig. 1 Photoresponsive sliding velocities of MTs in the presence of **1b** (error bars; s.e.m.). The concentration of **1b** corresponding to each curve is presented, except for 1 mM [circles for (a), squares for (b)]. (a) "No irra": prior to irradiation; "UV": irradiation with UV light after "No irra"; "Vis": irradiation with visible light after "UV". MTs bound to the glass surface were not observed in the following cases: Vis for 650 μM; no irra for 1 mM (n = 50 for 30 μM to 300 μM; n = 35 for 650 μM; n = 11 for 1 mM). (b) "UV 1st": irradiation with UV light; "Vis": irradiation with visible light after "UV 1st"; "UV 2nd": irradiation with UV light after "Vis". MTs bound to the glass surface were not observed in the following case: 1 mM, "Vis" [n = 50 for 30 μM to 300 μM; n = 35 (UV 1st), 12 (Vis), 18 (UV 2nd) for 650 μM; n = 17 (UV 1st) or 8 (UV 2nd) for 1 mM].

in their *trans* and *cis* forms. The average velocities of the MTs at 1 mM of *trans*-**1a** and *cis*-**1a** were 372 ± 7 and 383 ± 7 nm s^{-1}, respectively—approximately 60% of the value (*ca.* 660 nm s^{-1}) obtained for ATP at 1 mM, which was the sufficient concentration for saturating the sliding velocity of the MTs (Table 1). At 100 μM of **1a**, a concentration close to the value of K_m for ATP (85 μM, obtained through our motility assay; Fig. S3, ESI†), we recorded velocities of 179 ± 4 and 175 ± 3 nm s^{-1} for *trans*-**1a** and *cis*-**1a**, respectively—approximately 45% of the value for ATP (*ca.* 390 nm s^{-1}; Table 1). Thus, *trans*–*cis* isomerization of **1a** did not significantly affect the sliding velocity of the MTs. In contrast, for **1b**, which features a *tert*-butyl group at the azobenzene terminus, we observed a significant difference in the sliding velocities of the MTs before and after irradiation with UV light. The average velocity of the MTs at 100 μM *trans*-**1b** was 75 nm s^{-1} [*ca.* 20% of the velocity at 100 μM ATP (*ca.* 390 nm s^{-1}); Table 1 and Fig. 1a]. After converting *trans*-**1b** to *cis*-**1b** upon irradiation with UV light, the velocity reached 117 nm s^{-1} (an increase of 56%; Table 1 and Fig. 1a). Subsequent irradiation with visible light induced the reverse *cis*-to-*trans* isomerization of **1b**, resulting in a decrease in the velocity to 76 nm s^{-1}—almost the same value as that obtained initially prior to UV irradiation (Fig. 1a). We also observed this tendency—an increase in velocity after irradiation with UV light and a decrease with visible light—at other concentrations of **1b**. The NMR data shown in Fig. S2 (ESI†) suggest that about 10% of *trans*-**1b** remained at the photostationary state under UV irradiation. Complete isomerization would have achieved larger difference in the velocity change upon irradiation. When the concentration of *trans*-**1b** was too high (1 mM and "Vis" for 650 μM, Fig. 1a), we could not measure the velocities of the MTs because they were not bound to the kinesin-coated glass surface and drifted in the assay buffer. Therefore we could not measure or determine K_m for *trans*-**1b**, by the same token, or compare K_m for *trans*-**1b** to that for *cis*-**1b**.

To confirm the reproducibility and repeatability of the changes in the sliding velocities of the MTs upon irradiation with light, we inverted the order of the irradiation sequence. We observed the same tendency in the velocity changes as mentioned above when subjecting *cis*-**1b** ("UV 1st", Fig. 1b), itself obtained after irradiation at 365 nm, to irradiation at 436 nm, converting it into *trans*-**1b** ("Vis") with an accompanying decrease in the MT velocity, followed by irradiation at 365 nm to reform *cis*-**1b** ("UV 2nd"), accompanied by an increase. We could not measure the velocity of the MTs at 1 mM *trans*-**1b** (Fig. 1b, "Vis") for the same reason mentioned above.

Irradiation with UV light had no effect on the kinesin-induced motilities of the MTs in the presence of ATP (*i.e.* not the ATP-Azos) (Fig. S4, ESI†). Therefore, our observation of a different sliding velocity for each isomeric state of **1b** clearly reveals that the action of ATPase could be controlled dynamically through irradiation with light in the presence of the photochromic ATP analogue. We did not observe any significant changes in the sliding velocities of the MTs before or after UV light irradiation of **1a**, which lacks the *tert*-butyl group (Table 1). Thus, the nature of the substituent on the azobenzene unit of the ATP-Azo is a crucial factor affecting the photo-controlled functions of ATPases. The hydrophobic *tert*-butyl group presumably enhanced the difference in one or more of the properties (*e.g.* binding affinity, hydrolysis rate) of the *trans* and *cis* states of **1b** as substrates, relative to that of **1a**, ultimately resulting in observable changes in the motile velocities of the kinesin–MT–**1b** system.

As mentioned above, the sliding velocity of the MTs was clearly photo-controllable in the presence of **1b** at or below 300 μM (Fig. 1). At high concentrations of **1b**, however, we observed interesting behavior for the interactions between the MTs and the kinesin units coated on the glass surface, providing another approach toward photo-controlling the motility of the kinesin–MT system, as described below.

At 650 μM of *trans*-**1b**, the number of MTs attached to the glass surface was less than that at or below 300 μM; these MTs tended to glide on the kinesin-coated glass surface in a discontinuous manner or detach from the surface. In the presence of 1 mM of *trans*-**1b**, we observed only drifting MTs—that is, none were bound to the kinesin units coated on the surface (Fig. 2a). After converting *trans*-**1b** into *cis*-**1b** through UV light irradiation, the MTs were bound to and slid

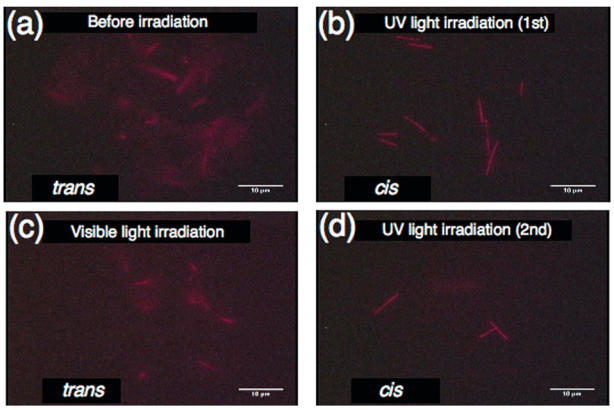

Fig. 2 Photoresponsive interactions between MTs and kinesins coated on the glass in the presence of 1 mM **1b**. (a) Prior to irradiation. (b) Irradiation with UV light after (a). (c) Irradiation with visible light after (b). (d) Irradiation with UV light after (c). Scale bar: 10 μm.

on the kinesin-coated surface (Fig. 2b). Subsequent sequential irradiation with visible and UV light resulted in the MTs detaching from and reattaching to (Fig. 2c and d) the surface, respectively. In contrast, for **1b** at or below 300 μM, we did not observe any clear difference in the attachment behavior of the MTs in the presence of *trans*-**1b** or *cis*-**1b** (Fig. S5, ESI†).

The exact mechanism of such attachment and detachment remains unclear, however, this phenomenon can be explained by considering that **1b** functions as a photoresponsive inhibitor of the intermolecular interactions between kinesin and the MTs in addition to behaving as a photoresponsive energy source when it is a substrate of ATPase kinesin. The amphiphilic nature of **1b**, with its hydrophilic triphosphate unit and hydrophobic *p*-*tert*-butylazobenzene moiety, suggests that it might form a complex with proteins *via* hydrophobic interactions, forming a triphosphate-presenting surface, at sufficiently high concentrations in water. We speculate that the formation of such complexes between *trans*-**1b** and either kinesin or the MTs inhibited the binding between the MTs and the kinesin units. The tendency of **1b** to form such complexes was confirmed by the observation of turbidity of the assay buffer solutions with *trans*-**1b** at 1 mM in the presence of proteins (*e.g.* casein) (Fig. S6, ESI†). The same solution became transparent after UV light irradiation inducing the photoisomerization of *trans*-**1b** to *cis*-**1b**; no such turbidity appeared for *trans*-**1b** at a concentration equal to or less than 300 μM (Fig. S6, ESI†).

In conclusion, we have realized reversible photo-control of a kinesin–MT motility system by using a novel photochromic ATP analogue, ATP-Azo **1b**. ATP-Azo works as both molecular gear and energy source, and, after further accomplishment of complete On/Off switch, would achieve higher regulation systems in bio-motor devices.[15] We anticipate that this technique might find wide applicability in nanobiotechnology, and furthermore, with other ATPases and related systems with other nucleotide triphosphates.[16]

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