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Photocontrol of Kinesin ATPase Activity Using an Azobenzene Derivative

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Azobenzene is a photochromic molecule that undergoes rapid and reversible isomerization between the *cis*- and *trans*-forms in response to ultraviolet (UV) and visible (VIS) light irradiation, respectively. Here, we introduced the sulfhydryl-reactive azobenzene derivative 4-phenylazophenyl maleimide (PAM) into the functional region of kinesin to reversibly regulate the ATPase activity of kinesin by photoirradiation. We prepared five kinesin motor domain mutants, A247C, L249C, A252C, G272C and S275C, which contained a single reactive cysteine residue in loops L11 and L12. These loops are considered to be key regions for the functioning of kinesin as a motor protein. PAM was stoichiometrically incorporated into the cysteine residues in the loops of the mutants. The PAM-modified S275C mutant exhibited reversible alterations in ATPase activity accompanied by *cis–trans* isomerization upon UV and VIS light irradiation. The ATPase activity exhibited by the *cis*-isomer of the PAM bound to the mutant was two times higher than that of the *trans*-isomer. Further, the PAM-modified L249C mutant exhibited reversible alterations in ATPase activity on UV–VIS light irradiation but exhibited the opposite effect on UV and VIS light irradiation. Using a photochromic azobenzene derivative, we have demonstrated that the ATPase activity of the motor protein kinesin is photoregulated.

Key words: ATPase, kinesin, motor protein, photochromic molecule, photocontrol.

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol *bis* (b-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid; KIF5a, mouse brain kinesin; LDH, lactate dehydrogenase; L5, loop 5; L11, loop 11; L12, loop 12; MT, microtubule; NADH, nicotinamide adenine dinucleotide; PK, pyruvate kinase; PEP, phosphoenolpyruvic acid; SDS, sodium dodecyl sulfate; SH, sulfhydryl; WT, wild-type.

Photochromic molecules can undergo a reversible configurational change upon light irradiation; consequently, they have applications in dimmer materials, optical storage materials, indicator materials, *etc*. (*1–3*). Of late, the application of photochromic molecules to functional biomolecules has received increasing interest. The potential applications of these biomolecules include use in biosensors, photocontrol of industrial enzymes, medical treatment and the study of cell functions. Azobenzene, a widely studied photochromic compound, can be reversibly isomerized between the *cis*- and *trans*-forms by ultraviolet (UV) and visible (VIS) light irradiation, respectively (*4*, *5*). Interestingly, the *cis–trans*-isomerization of an azobenzene derivative cross-linked to an α-helical synthetic peptide induced by UV/VIS photoirradiation has been demonstrated to induce a reversible change in the secondary structure of the peptide—from a helical to a random coil structure, and vice versa (*6*, *7*). It has also been reported that the incorporation of photochromic groups into enzymes photomodulates their activity, as in the case of papain and glucose oxidase (*8*, *9*). We have previously demonstrated the possible application of an azobenzene derivative in regulating a conformational change in skeletal muscle myosin. The azobenzene derivative, namely, 4,4′-azobenzene-dimaleimide (ABDM), was incorporated into the SH1–SH2 region of skeletal muscle myosin subfragment-1 (S1), which is a potential energy-transducing site. Myosin S1 was modified with ABDM, and the global conformational change of S1 induced by the *cis–trans*-isomerization of the crosslinked ABDM in response to UV/VIS light was investigated. It was demonstrated that the *cis–trans* isomerization of ABDM promotes a swing in the lever arm of S1 in a direction opposite to that induced by ATP binding (*10*).

Kinesin is the ATP-driven motor protein that plays important physiological roles in intracellular transport, mitosis and meiosis, control of microtubule (MT) dynamics and signal transduction. Recent structural analysis of kinesin (*11–14*) has revealed that kinesin is strikingly similar in structure to the catalytic domains of myosin. This similarity suggests that the motor proteins and molecular switches, which utilize the energy from ATP for motility, use a similar conformational strategy during the first stage of energy transduction. Since kinesin mutants can be readily prepared using *Escherichia coli*, their expression system, structure and functions have been well studied. The several loops in the molecular structure of kinesin have been

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demonstrated to play a significant role in motility (*15–18*). Loops L11 and L12, which are located at the MT-binding site are the potential key regions responsible for motility (*19–23*). Therefore, it is believed that the incorporation of photochromic molecules into the functional region will enable the regulation of kinesin. In the present study, kinesin mutants containing a single cysteine residue in either L11 or L12 were prepared for the introduction of an azobenzene derivative. We successfully demonstrated that the ATPase activities of the kinesin proteins modified with an azobenzene derivative could be regulated by UV–VIS light irradiation.

MATERIALS AND METHODS

Ligation enzymes were purchased from Toyobo Co., Ltd, unless stated otherwise. Oligonucleotides were obtained from Sigma Genosys. The apparatus for Talon Superflow metal affinity chromatography was procured from Clontech. Chemicals reagents were purchased from Wako Pure Chemicals unless stated otherwise. Tubulin was purified from porcine brain as described by Hackney (*24*).

Expression and Purification of Mouse Kinesin Mutants L249C and S275C—Site-directed mutagenesis of kinesin was carried out in *E. coli* by using the self-ligation method with the following primers (mutated codons are underlined): KIFL249C5′, 5′-GTTTGCGACGAGGCAAAG AATATCAACAAG-3′; KIFL249C3′, 5′-GGCTCCCTCTGC CCCTGTCTTGCTGACCTT-3′; KIFS275C5′, 5′-TGCTAC GTGCCGTACCGCGACAGCAAAATG-3′ and KIFS275 C3′, 5′-TTTGGTGCCCTCTGCCAGTGCAGAGATCAC-3′. The background construct used was pET15b : K334-Cys, a pET15b-based plasmid in which Cys8, 66, 169 and 296 were mutated to Ser. Following DNA sequence confirmation of the additional desired probe mutation using a 3100-Avant DNA Analyzer (ABI PRISM), the plasmid was transformed into the *E. coli* strain BL21 (DE3) for expression. The mutant kinesin was purified using Talon Superflow metal affinity chromatography. The resin was equilibrated with NMM buffer [300 mM NaCl, 20 mM, morpholinopropane sulphonic acid (MOPS) (pH 7.0), 1 mM $MgCl_2$, 0.1 mM ATP and 0.2 mM β-mercaptoethanol] and the supernatant was added to absorb the expressed protein. The expressed kinesin was eluted with 150 mM imidazole–HCl in NMM buffer, and its purity was assessed by SDS-PAGE, which revealed a single band on Coomassie-stained gels. The samples were dialysed against 120 mM NaCl, 2 mM $MgCl_2$, 0.1 mM ATP, 30 mM Tris–HCl pH 7.5 and 1 mM dithiothreitol (DTT), and stored at −80°C until further use.

Photoirradiation for Isomerization of 4-Phenyazomaleinail—The isomerization of 4-phenylazophenyl maleimide (PAM) was performed at 0°C using UV-light irradiation generated by a Blak-Ray lamp (16 W) (UVP Inc., San Gabriel, USA) at 366 nm for induction of the *cis*-state and by VIS light irradiation using a room fluorescent lamp (27 W) for induction of the *trans*-state.

Modification of Mutated Sites in Kinesin using PAM—The modification of the mutated sites in kinesin using PAM was carried out by reacting 20 μM kinesin and 100 μM PAM in the presence of 120 mM NaCl, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 3% *N*, *N*′-dimethylformamide (DMF) and 1 mM ADP for 10 min at 25°C. The reaction was terminated by the addition of DTT to a final concentration of 5 mM. The modified kinesin was isolated from the unreacted reagents by using a gel filtration column (10 DG; Bio-Rad, Hercules, CA, USA) pre-packed with 120 mM NaCl, 30 mM Tris–HCl (pH 7.5) and 2 mM $MgCl_2$. The stoichiometry of the incorporated PAM in comparison to that of kinesin was determined based on the absorption spectrum obtained using an extinction coefficient of 10,620 $M^{-1} cm^{-1}$ at 350 nm for the PAM group.

Measurement of the ATPase Activity of Kinesin Following Modification with PAM and Photoirradiation—The MT-dependent ATPase activity of kinesin was measured at 25°C in a reaction mixture comprising PAM-kinesin, 50 mM imidazole–HCl (pH 6.7), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM ethylene glycol *bis* (β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid (EGTA), 1 mM β-mercaptoethanol, 0.2–10 μM MT and 1 mM ATP. The reaction was terminated by the released phosphate (Pi) that was measured according to the method described by Youngburg and Youngburg (*25*). The basal ATPase activity of kinesin was measured using the established coupled reaction with nicotinamide adenine dinucleotide (NADH) (*26*) at 25°C in a solution of 50 mM imidazole–HCl (pH 6.7), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM β-mercaptoethanol, 80 μM NADH, 800 μM phosphoenolpyruvic acid (PEP), 47 μg/ml pyruvate kinase (PK), 12 μg/ml lactate dehydrogenase (LDH) and 25 μM ATP.

RESULTS

ATPase Characterizations of Kinesin Mutants Containing Cysteine in either L11 or L12—In order to incorporate the photochromic molecule into either the L11 or L12 loop, kinesin mutants A247C, L249C, A252C, G272C and S275C containing a single cysteine in the loops were prepared according to the established methods as described in MATERIALS AND METHODS section. The crystal structures of the amino acid residues that were substituted by cysteine are shown in Fig. 1. The ATPase activities of the kinesin mutants were measured in the presence of various concentrations of MT, in order to determine whether the mutants had retained normal enzymatic properties. The maximal ATPase activity and the MT concentration for half-maximal stimulation of the kinesin mutants are summarized in Table 1. The MT-dependent ATPase activities of S275C, G272C and A247 were slightly lower than that of wild-type (WT) kinesin, indicating that the mutants retained normal enzymatic properties. In contrast, the V_{max} of ATPase activity in the case of L249C was reduced by 20% compared to that of the WT, indicating that the substitution of Leu249 in L11 to Cys leads to the direct effect on the ATPase site. The K_{MT} values of L249C and A252C were approximately three times higher than that of the WT, indicating that the two amino acids are directly involved in MT binding.

Modification of Kinesin Mutants with PAM—We employed PAM as a sulfhydryl (SH)-group-reactive

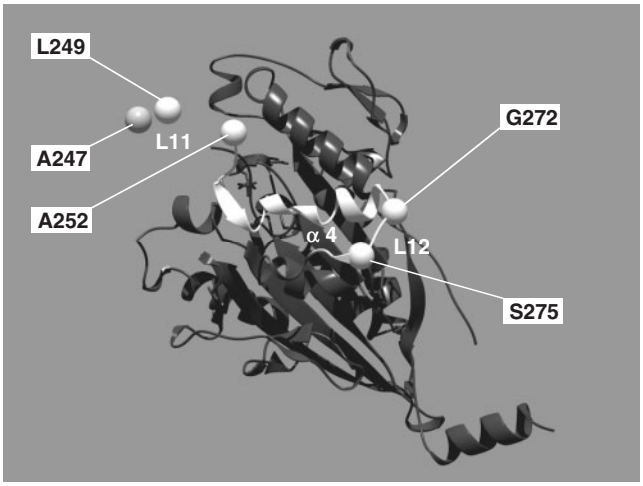

Fig. 1. **Location of the amino acids substituted by cysteine, in order to introduce PAM in the crystal structure of the kinesin motor domain.** The L12 region is shown in white, and the α-carbons of the amino acids G272 and S275 substituted by cysteine are indicated in the space-filling model. The 3D structure was prepared using the molecular graphics program Mol Feat by using the coordinate data (2KIN) of *Rattus norvegicus* kinesin from the protein data bank database. Since the structure of L11 has not been described in the crystal structure of the coordinate data (2KIN), the putative location of A247, L249 and A252 are indicated based on a comparison with the structure of the human ubiquitous kinesin (1BG2). ADP is shown in stick representation.

Table 1. **Basal ATPase and calculated V_{max} and K_{MT} values.**

	V_{max} (s^{-1})	K_{MT} (μM)	Basal ATPase (s^{-1})
WT Cys lite	38.42 ± 2.06	0.90 ± 0.17	0.023 ± 0.001
G272C	33.62 ± 1.97	0.83 ± 0.171	0.005 ± 0.001
G272C-PAM *cis*	21.89 ± 0.81	0.46 ± 0.07	0.005 ± 0.001
G272C-PAM *trans*	20.00 ± 0.88	0.44 ± 0.08	0.004 ± 0.001
S275C	21.42 ± 2.13	0.79 ± 0.28	0.023 ± 0.004
S275C-PAM *cis*	16.39 ± 0.58	1.46 ± 0.16	0.011 ± 0.003
S275C-PAM *trans*	7.49 ± 0.30	1.54 ± 0.18	0.007 ± 0.001
A247C	37.47 ± 2.75	1.34 ± 0.31	0.004 ± 0.001
A247C-PAM *cis*	27.33 ± 1.36	0.87 ± 0.15	0.002 ± 0.001
A247C-PAM *trans*	24.67 ± 1.27	0.83 ± 0.15	0.004 ± 0.001
L249C	8.54 ± 0.60	3.41 ± 0.57	0.021 ± 0.004
L249C-PAM *cis*	2.73 ± 0.21	6.91 ± 0.99	0.012 ± 0.002
L249C-PAM *trans*	3.21 ± 0.16	5.06 ± 0.55	0.019 ± 0.002
A252C	21.29 ± 1.20	3.05 ± 0.43	0.014 ± 0.004
A252C-PAM *cis*	<0.9	ND	ND
A252C-PAM *trans*	ND	ND	ND

V_{max} and K_{MT} were estimated from the MT-dependent ATPase data by using the formula $V = V_{max}/(1 + K_{MT}/[\text{microtubules}])$. Basal ATPase activities were measured at 25°C in a reaction mixture comprising 1 μM kinesin, 50 mM imidazole–HCl (pH 6.7), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM β-mercaptoethanol, 80 μM NADH, 800 μM PEP, 47 μg/ml PK, 12 μg/ml LDH and 25 μM ATP. ATPase activity was measured from three independent protein preparations. Data are presented as mean ± SD.

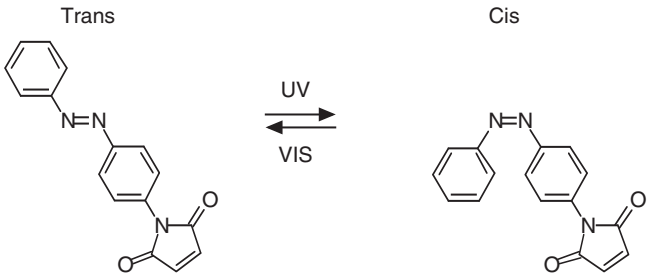

Fig. 2. **Schematic representation of the photoisomerization of PAM.** The molecular structures of *cis*- and *trans*-PAM. UV light irradiation converts the *trans*-form of PAM into the hydrophilic *cis*-form. VIS light irradiation reverses the conversion.

photochromic molecule (Fig. 2), in order to incorporate photochromic molecules into the MT-binding region of kinesin. The configurational state (*cis* or *trans*) of azobenzene and its derivatives can be monitored by UV/VIS light absorption spectroscopy. The UV/VIS absorption spectrum for PAM was similar to that of azobenzene. Figure 3 shows the absorption spectrum of the *trans*-form of PAM in a solution of DMF. The absorption maximum of *trans*-PAM was recorded at 330 nm, whereas azobenzene exhibited maximum absorbance at 315 nm under the same conditions. The irradiation of the *trans*-PAM solution by UV light (366 nm) generated using a Blak-Ray lamp (16 W) subsequently led to a marked reduction in the peak at 330 nm. Following irradiation for 5 min, the alteration of the spectrum was saturated. On comparison with the published spectral data (*27*), the solution was estimated to contain approximately 75% *cis* and 25% *trans*-forms of PAM.

In order to identify the optimal conditions for the specific labeling of the cysteine residues in L11 or L12, we conducted time-course studies in order to determine the reaction- and concentration-dependence of PAM for the kinesin mutants. Figure 4A shows the relationship between ATPase activity and modification time for S275C at 25°C when the amount of PAM is 5-fold greater than the amount of kinesin. In this experiment, PAM was used without light irradiation that resulted in the formation of the *trans*-isomer. Within 30 s, the ATPase activity was reduced, modified and saturated at 20% of the intact S275C activity. The incorporation of PAM into S275C was saturated when the amount of PAM was 2.5-fold higher than the amount of kinesin in the reaction solution (Fig. 4B). PAM was almost stoichiometrically incorporated into S275C. PAM modified other mutants in a manner similar to that of S275C in the time-course and concentration-dependence reactions (data not shown). As shown in Fig. 5, the spectral change in the UV/VIS absorbance in the case of kinesin mutant S275C incorporated with PAM was similar to that of free PAM, indicating the reversible isomerization between the *cis*- and *trans*-forms. The other mutants modified with PAM also exhibited reversible spectral change on UV–VIS light irradiation.

Photocontrol of the ATPase Activities of the PAM-kinesin Mutants—The alteration of the ATPase activity of kinesin mutants modified by PAM accompanied by the *cis–trans* isomerization of PAM was examined.

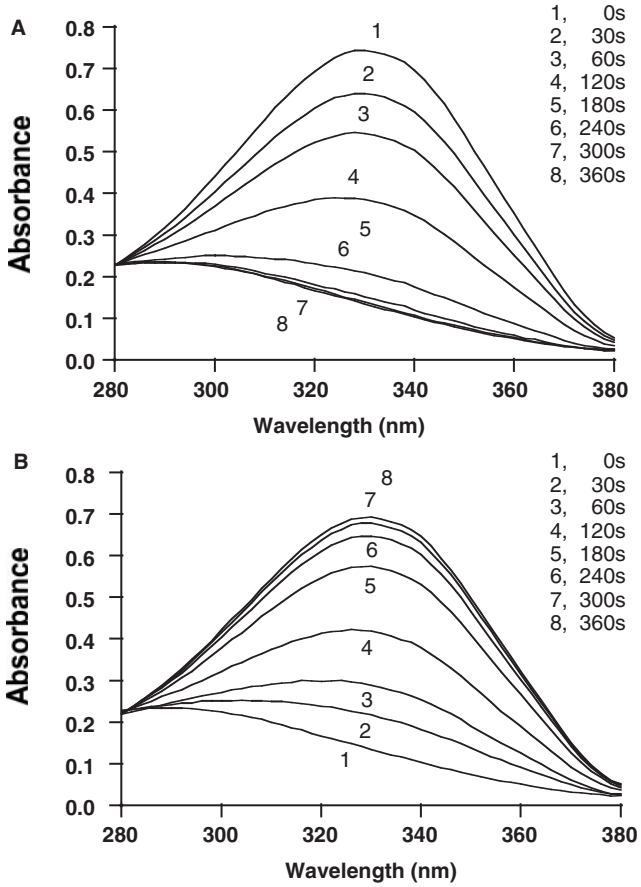

Fig. 3. **Changes in the absorption spectrum of free PAM induced by UV and VIS light irradiation.** (A) Free PAM (30 μM) in 100% DMF was irradiated using a UV lamp (Blak-Ray lamp, 16 W) at 366 nm for 15, 30, 60, 120, 180, 240, and 300 s at room temperature. (B) Free PAM (30 μM) in 100% DMF irradiated using UV lamp (Blak-Ray lamp, 16 W) at 366 nm for 300 s was subsequently irradiated using fluorescent room light (27 W) for 15, 30, 60, 120, 180, 240 and 300 s at room temperature.

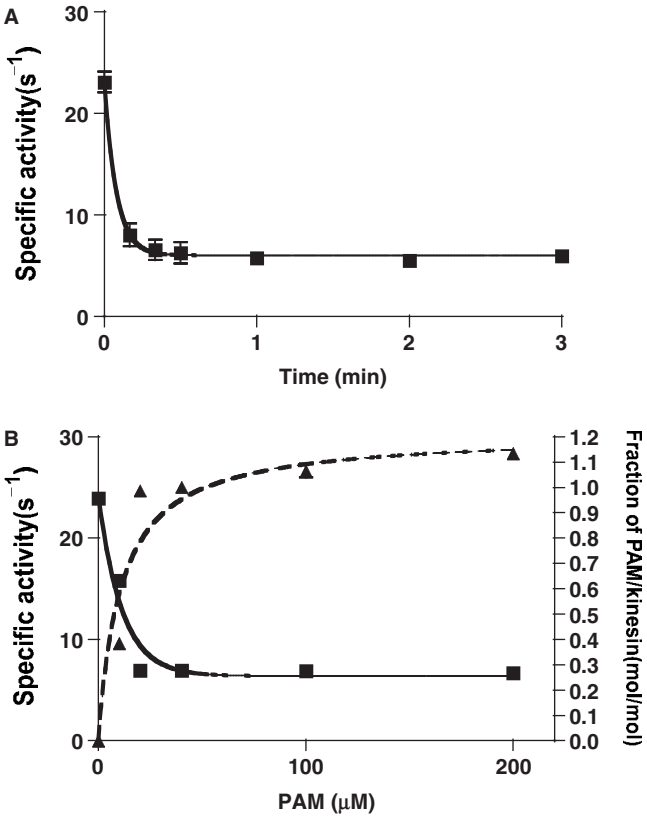

Fig. 4. **(A) The measurement of ATPase activity during the time-course experiment involving the incorporation of PAM into the S275 of kinesin.** Twenty micromolar kinesin was modified using a 5-fold molar excess of PAM at 25°C in a buffer containing 120 mM NaCl, 2 mM $MgCl_2$, 1 mM ADP, 3% DMF, 30 mM Tris–HCl, pH 7.5 for 0, 20, 30, 60, 120, and 180 s. The reaction was terminated by the addition of 5 mM DTT. Subsequent procedures were performed as described in MATERIALS AND METHODS section. (B) The measurement of ATPase activity during the concentration-dependent incorporation of PAM into the S275 of kinesin. Twenty micromolar kinesin was reacted for 10 min with 10, 20, 50, 100 and 200 mM PAM at 25°C in a buffer containing 120 mM NaCl, 2 mM $MgCl_2$, 1 mM ADP, 3% DMF, 30 mM Tris–HCl, pH 7.5. The reaction was terminated by the addition of 5 mM DTT. The ATPase activity of kinesin was measured at 25°C in a reaction mixture comprising kinesin, 50 mM imidazole–HCl (pH 6.7), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM β-mercaptoethanol, 5 μM MT and 1 mM ATP.

Figure 6 shows the MT-concentration dependence of the ATPase activity of kinesin mutants S275C (A) and L249C (B) modified with PAM irradiated by VIS or UV light irradiation. On irradiation with UV light—conditions under which the PAM *cis*-forms were dominant—PAM-S275C exhibited approximately 2-fold higher maximum activity than PAM-S275C irradiated with VIS light, where the PAM *trans*-forms were dominant. In contrast, in L249C, the VIS light irradiation induced a 20% higher ATPase activity. For the other mutants, no significant alterations in ATPase activity correlated with the *cis–trans* isomerization of PAM were observed (Table 1).

The alterations in the ATPase activity of S275C-PAM and L249C-PAM correlated with the *cis–trans* isomerization were completely reversible (Fig. 7). Even after providing alternating UV–VIS light irradiation three times, the reversible alteration of ATPase activity was retained. The apparent K_{MT} of the *cis*- and *trans*-states were almost the same for both the mutants modified with PAM, indicating that the *cis–trans* isomerization did not affect MT binding. Therefore, the alteration of the ATPase activity might be due to the allosteric effect of PAM on the ATPase site. From these experimental data, it has been clearly demonstrated that the ATPase activity of kinesin modified by PAM is reversibly controlled by UV–VIS light irradiation.

DISCUSSION

The aim of this study was to control the functioning of the kinesin motor protein by using photochromic compounds. It has been previously demonstrated that the motor domain of kinesin is very similar to that of myosin. It has also been suggested that these two motor proteins

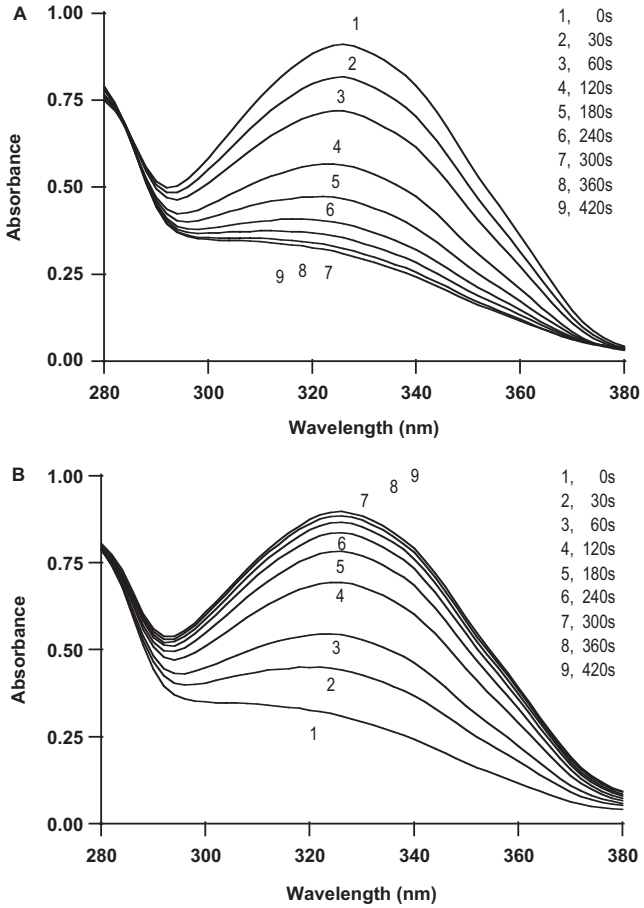

Fig. 5. **Change in the absorption spectrum of S275C-PAM induced by UV and VIS light irradiation.** (A) S275C-PAM (30 μM) was irradiated using a UV lamp (Blak-Ray lamp, 16 W) at 366 nm for 15, 30, 60, 120, 180, 240, 300 and 420 s at room temperature in a solution of 120 mM NaCl, 30 mM Tris–HCl (pH 7.5) and 2 mM $MgCl_2$. (B) S275C-PAM irradiated using a UV lamp (Blak-Ray lamp, 16 W) at 366 nm for 300 s was subsequently irradiated using fluorescent room light (27 W) for 15, 30, 60, 120, 180, 240, 300 and 420 s at room temperature.

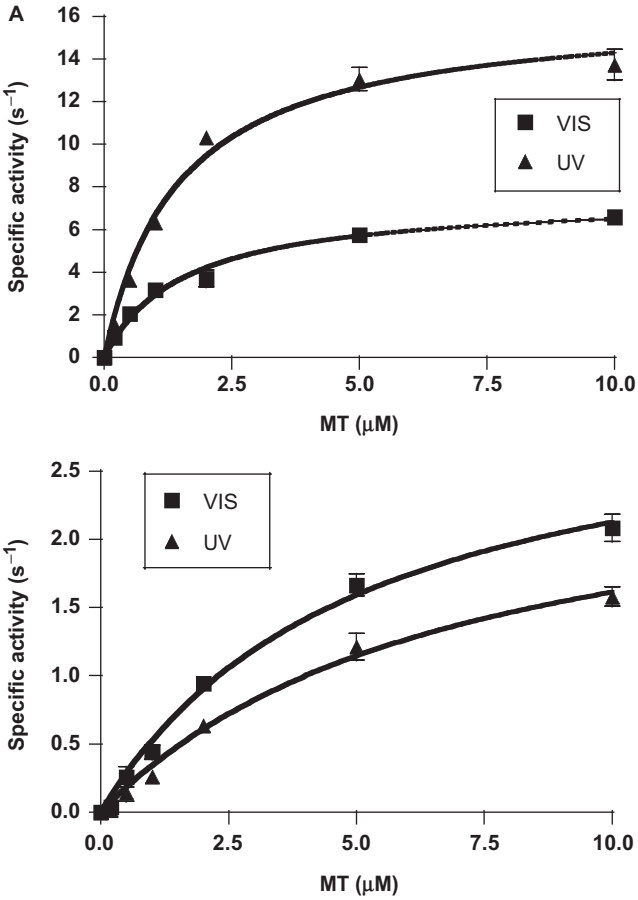

Fig. 6. **The effect of UV–VIS light irradiation on the MT-dependent ATPase activity of kinesin mutants S275C (A) and L249C (B) modified with PAM.** The ATPase activity of kinesin was measured at 25°C in a solution of 50 mM imidazole–HCl (pH 6.7), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM ATP and various concentrations of MTs. For conversion to the *cis*-state (close triangles), the kinesin mutants modified with PAM were irradiated using a UV lamp for 5 min on ice prior to the ATPase assay; for conversion to the *trans*-state (closed squares), the kinesins were irradiated using fluorescent room light for 5 min on ice prior to the ATPase assay.

might share a common energy-transducing mechanism. Recent X-ray crystallographic (*11–14*) and point mutation studies of the motor proteins (*28*, *29*) have provided important information regarding mechanical transduction, suggesting that the chemical energy of ATP is mechanically transmitted to generate the physical swing of the lever arm or neck-linker similar to a camshaft motion through subdomain steric interactions. Therefore, we expected that the introduction of a switching device, such as a photochromic molecule, into the energy-transducing region might regulate the motor protein. Previously, using an azobenzene derivative and then crosslinking it between SH1- and SH2-reactive cysteine residues, we successfully induced lever arm swinging in skeletal muscle myosin by influencing the *cis–trans* isomerization (*10*).

In the present study, we focused on kinesin as the target motor protein for the application of photochromic molecules. Since engineered kinesin mutants are easily expressed in *E. coli*, their structure and enzymatic properties have been well characterized. Using point mutations, fluorescent probes and electron spin resonance, we have previously established the functional importance of the L11 and L12 of kinesin. Therefore, we prepared kinesin mutants containing a single cysteine in each loop for modification with PAM. It has been demonstrated that L11 is one of the MT-binding regions connected to switch II that is believed to be a relay helix corresponding to that of myosin. For L11, we prepared three mutants, A247C, L249C and A252C. A247 and L249 are located in the middle of the loop, and A252 lies in the neck of the loop linking to the α4 helix (Fig. 1). The ATPase activity of L249C was significantly reduced by modification but was reversibly regulated via UV–VIS light irradiation. As shown in Fig. 7, the alteration of the ATPase activity correlated with *cis–trans* isomerization

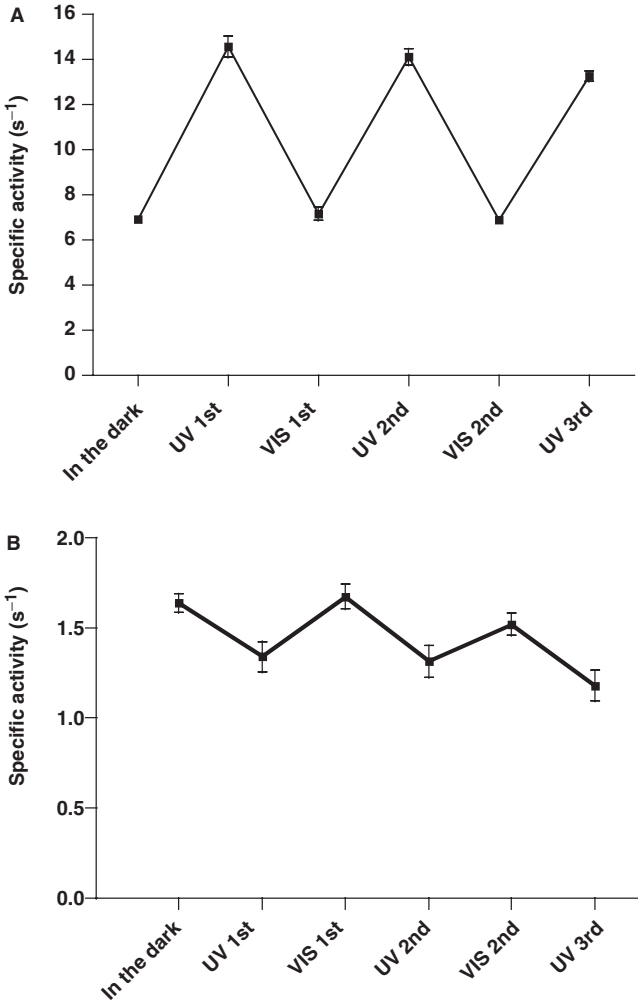

Fig. 7. **Reproducible reversibility of the photocontrol of ATPase activity of the kinesin mutants modified with PAM.** The kinesin mutants PAM-S275C (A) and PAM-L249C (B) were irradiated by UV and VIS light alternately prior to ATPase assays under the same conditions described in the legend for Fig. 6. The alternating UV–VIS light irradiation was repeated three times. The ATPase activity was measured in three independent protein preparations. Error bars indicate SD.

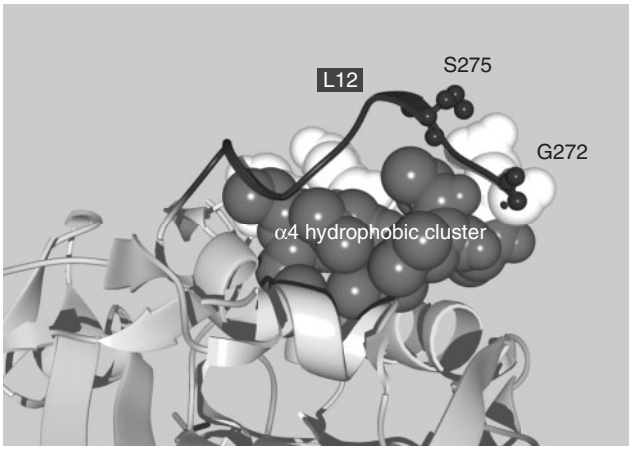

Fig. 8. **Location of the amino acids substituted by cysteine in L12 and the hydrophobic cluster of α4.** The L12 region is shown as a black ribbon, and the amino acids S275 and G272 substituted by cysteine are indicated in the ball and stick model. The hydrophobic and hydrophilic amino acids in α4 are shown in dark grey and white space-filling models, respectively. The 3D structure was prepared using the molecular graphics program Mol Feat by using the coordinate data (2KIN) of *R. norvegicus* kinesin from the protein data bank database. In the crystal structure (2KIN), the 275th amino acid shown in ball and stick is threonine that corresponds to the serine of the kinesin prepared in this study.

was reversible and highly reproducible. Modification of A252C with PAM almost completely abolished the ATPase activity. On the other hand, modification of A247C with PAM does not significantly affect ATPase activity and MT binding. Therefore, it is believed that the region of L11 containing L249 and A252 connecting to α4 is crucial for ATPase and binding. The neck region in the vicinity A252 of L11, in particular, may play an important role in transducing the signal of MT binding to switch II. Based on cryo-electron microscopic observation, Sindelar and Downing (*21*) also suggested that the connector region plays an important role in assisting energy transduction. The direct incorporation of photochromic molecules into the crucial region should therefore be avoided. The N-terminal segment of L11 may be a candidate for further study.

L12 is another significant MT-binding region, connected to switch II (*21*). We introduced cysteine into the middle of L12 at positions G272 and S275. G272 is located in the neck of the loop connecting to the α4 helix and S275 is located in the middle of the loop. The kinesin mutant G272C modified with PAM did not exhibit the UV–VIS light irradiation-dependent alteration in ATPase activity. On the other hand, S275C modified with PAM did exhibit UV–VIS light irradiation-dependent alteration in ATPase activity. Interestingly, UV and VIS light irradiation had opposite effects on S275C and L249C—the *cis*- and *trans*-conformational changes induced by PAM were different at S275 and L249.

As shown in Table 1, the K_{MT} of the mutants modified with PAM did not undergo a change when irradiated by UV and VIS light. Therefore, the localized, small conformational change induced by the *cis–trans* isomerization of PAM at the MT-binding region may not be sufficient to control binding. However, it is probable that the *cis–trans* isomerization induces conformational changes at the ATPase site in an allosteric manner to alter the ATPase activity. This supposition is supported by the alteration of the basal ATPase activities due to UV–VIS light irradiation (Table 1). As we were unable to observe the steric structure of L11 due to structural ambiguity, it is difficult to discuss the possible mechanism of photocontrol of PAM-modified kinesins at the molecular level. However, for the kinesin mutants modified by PAM at L12, we suggest the following possible explanation for the isomerization-dependent alteration in ATPase activity based on the steric structure. As shown in Fig. 8, L12 is located along the

cluster of hydrophobic amino acid side chains in α4 and the hydrophilic side chain of S275 protrudes outwardly from the opposite side of the hydrophobic cluster. It is known that the hydrophobicity of *trans*-azobenzene is considerably higher than that of *cis*-azobenzene (*30*). The *trans*-azobenzene moiety of PAM bound to S275 interacts with the hydrophobic cluster in α4, thereby inducing a distortion of L12. The distortion of L12 modulates ATPase activity. In contrast, the less hydrophobic *cis*-PAM bound to S275 interacts slightly with the hydrophobic cluster and induces a small distortion of L12. As shown in Table 1, the order of the ATPase activity, S275C > S275C-PAM*cis* > S275C-PAM*trans* corresponds to the degree of hydrophilicity. On the other hand, as G272 is located in the neck region of L12 connecting to α4 helix, *cis–trans* isomerization may not induce a distortion of L12.

We have performed preliminary stopped-flow experiments to demonstrate the effect of the *cis–trans* isomerization of PAM-modified S275C on the ATPase kinetic pathway. The results indicate that the binding rate and affinity of ATP for PAM-S275C were not altered by *cis–trans* isomerization. However, the rate of ADP release ($0.7 \times 10^{-3}\,s^{-1}$) from *trans*-PAM-S275C was apparently slower than that ($1.0 \times 10^{-3}\,s^{-1}$) of *cis*-PAM-S275C. This suggests that the distortion of L12 induced by *trans*-PAM at S275 may stabilize the kinesin-ADP state.

The results indicate that the modification of the appropriate site in the loops by photochromic molecules is crucial. The conformational change and the molecular size of the photochromic molecules are also important. The molecular size (5–15 Å) and the hydrophobic–hydrophilic properties of the PAM azobenzene derivative employed in this study change dramatically, concomitant with *cis–trans* isomerization (*30*). The molecular design of the photochromic molecules should be considered in order to ensure their conformational compatibility with the structure of the functional region. In our preliminary study, we employed the derivatives of another photochromic molecule, spiropyran. The spiropyran ring opens to yield ionized $–N^+$ and $–O^-$ ions on UV light irradiation and closes to yield a nonpolar hydrophobic structure (*31–34*). One of the spiropyran derivatives produced a photoregulatory effect on kinesin ATPase similar to that of azobenzene shown in this study. The spiropyran compound undergoes photoisomerization without undergoing a significant change in its molecular size. Therefore, the alteration of the kinesin ATPase might be dependent mainly on the change in the polarity of the photochromic molecule bound to the functional region.

The technique of coupling point mutations and the incorporation of photochromic molecules into the functional site is also applicable to other functional proteins. We have successfully performed preliminary photocontrol of the binding of calmodulin to a target peptide using PAM. Calmodulin modified with PAM near the EF-hand in the N-terminal domain binds to the target peptide M13 on UV light irradiation and dissociates on VIS light irradiation in pCa 6.25 (unpublished data).

In conclusion, we succeeded in photocontrolling the ATPase activity of kinesin using a photochromic compound. The technique may also be applicable to other functional biomolecules. It is expected that further studies in this field will contribute to the development of photodriven or photoregulated bionanomachines.

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