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Intermolecular Cross-Linking of a Novel Rice Kinesin K16 Motor Domain with a Photoreactive ATP Derivative

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A fluorescent photoreactive ATP derivative, 2′(3′)-*O*-(4-benzoylbenzoyl)-1,N^6-*etheno*-ATP (Bz_2-εATP), was synthesized and reacted with the rice kinesin K16 motor domain (K16MD). In the presence of ADP or ATP, UV irradiation of the K16MD solution containing Bz_2-εATP resulted in a new 100 kDa band, which was an intermolecular cross-linked product of motor domains. In contrast, no cross-linking was observed in the absence of nucleotides. For the motor domain of mouse brain kinesin and skeletal muscle myosin subfragment-1, no such intermolecular photo cross-linking by Bz_2-εATP was observed. Our results indicate that Bz_2-εATP acts unusually as a photoreactive crosslinker to detect conformational changes in K16MD induced by nucleotide binding resulting in the formation of dimers.

Key words: conformational change, crosslink, photo reactive ATP derivatives, rice kinesin.

Abbreviations: Bz_2-εATP, 2′(3′)-*O*-(4-benzoylbenzoyl)-1,N^6-*etheno*-ATP; DTT, dithiothreitol; DMF, dimethylformamide; εATP, 1,N^6-*etheno*-ATP; EDTA, ethylendiaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid; HPLC, high performance liquid chromatography; K16MD, rice kinesin K16 motor domain; KIFs, kinesin super families; Mant-ATP, 3′-*O*-(*N*-methylanthraniloyl)-ATP; MT, microtubule; NBD-ATP, 2′(3′)-*O*-[6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic]-ATP; Ni-NTA, nickel nitrilo triacetic acid; PCR, polymerase chain reaction; P_i, phosphate; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

Kinesins constitute a superfamily of ATP-driven microtubule motor proteins (KIFs) found in all eukaryotic organisms. Kinesins are involved in many diverse cellular functions such as the transportation of organelles and vesicles, spindle formation and elongation, chromosome segregation during cell division, microtubule dynamics, and morphogenesis (*1–5*). Kinesin superfamilies in animals have been well studied, especially those from mice and humans (*6–11*). In contrast, only a few kinesins have been characterized in plants, including *Arabidopsis thaliana* (*12–16*). Recently, we have succeeded in expressing the motor domain of rice plant–specific kinesin K16 (*17*). The enzymatic features of this kinesin have been studied. The motor domain of the rice kinesin has different characteristics with regard to its affinity for ADP and microtubules in comparison with conventional kinesins (*17*). However, rice kinesin K16 retains enzymatic properties even after the removal of the bound ADP from the ATPase site, in contrast to conventional kinesins (unpublished data).

Photoreactive ATP analogues have been successfully used to identify ATP binding proteins of cells and some purified protein kinases (*18–20*). Especially for myosin ATPase, several kinds of photoreactive ATP analogues have been used to identify ATP binding sites and to investigate conformational changes in the ATP binding sites (*21–23*). Bz_2-εATP is a fluorescent photoreactive ATP analogue utilized to investigate myosin by Cremo *et al*. (*24*). The ATP analogue was incorporated into the ATP binding site of skeletal muscle myosin with high efficiency, and the fluorescence of the incorporated ATP analogue was utilized to monitor conformational changes in the ATP binding site induced by actin binding (*25*).

To our knowledge, no such a biochemical approach using photoreactive ATP analogues has been performed for kinesin. In the process of studying the ATP binding site of rice kinesin using several kinds of ATP analogues, we encountered an interesting phenomenon: the motor domain of rice kinesin was unusually cross-linked by a mono-functional photoreactive fluorescent ATP analogue, Bz_2-εATP, in the presence of nucleotides. The cross-linking was observed only on the rice kinesin motor domain, but not on other conventional kinesin motor domains. The novel observation of cross-linking by a photoreactive ATP analogue may reflect the unique properties of rice kinesin. This is the first biochemical approach to detect conformational changes in the novel plant-specific kinesin motor domain using a photoreactive fluorescent ATP analogue.

MATERIALS AND METHODS

Synthesis of 3′(2′)-O-(4-Benzoylbenzoyl)-1,N^6-Etheno-adenosine 5′-Triphosphate (Bz_2-εATP)—Bz_2-εATP was

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synthesized according to the method of Cremo and Yount (*24*). Bz_2 acid (1 mmol) and carbonyldiimidazole (2 mmol) were stirred for 20 min at room temperature in 2.4 ml of dry DMF. εATP (0.2 mmol) was dissolved in 3 ml of water and then added dropwise to the reaction mixture. The coupling reaction was allowed to proceed for 24 h at room temperature in the dark under continuous stirring. Thirty volumes of chilled acetone (–20°C) were then added, and the precipitate was collected by centrifugation (1,630 × *g*) for 20 min at 4°C, and dissolved in 3 ml of 0.1% TFA. The product was purified by high performance liquid chromatography (HPLC) on an RP-C18 column. Elution was carried out with a linear acetonitrile gradient (0 to 90%) in 0.1% TFA at a flow rate of 1 ml/min. The elution profile was monitored by the absorbance at 300 nm. The purity of the product was analyzed by thin-layer chromatography (TLC) on silica gel plates (Silica Gel-70 F254; Wako Pure Chemical, Osaka, Japan). A single spot of Bz_2-εATP was observed. Mass spectral analysis of the product revealed a mass identical to that of Bz_2-εATP, and the UV spectrum showed a major peak of ATP at 260 nm and a minor peak of ethenoadenosine at 275 nm. The fluorescence excitation and emission maximum were 275 nm and 410 nm, respectively.

Preparation of the Rice Kinesin K16 Motor Domain—K16 plasmid was a gift from the National Institute of Agrobiological Science (NIAS). A DNA fragment encoding amino acids 77–419 of the predicted 343 residue K16 motor domain was synthesized by the polymerase chain reaction (PCR) from the LambdaFLC-K16 plasmid using the forward and reverse primers 5′-CAAAGCTAGCGACCCGGCGCCCAAGGAGAATGTCA-3′ and 5′-CAAACTCGAGCTTTATTAAAGATTTTTCATCTATAA-3′, respectively. The 5′-PCR oligonucleotide consisted of *Nhe*I and corresponded to eight amino acids (D77–V84) of the K16 protein. The 3′-PCR oligonucleotide consisted of 24 bases corresponding to amino acids I412–K419 of the K16 protein followed by a *Xho*I site. Four extra bases were added to each primer at the restriction site to enhance binding by the *Nhe*I and *Xho*I restriction sites. The gel-purified PCR fragment was cloned into the pET21a expression vector using the *Nhe*I and *Xho*I restriction sites. DNA sequencing was performed by the dideoxy chain termination method with a SQ-5500 sequencer (Hitachi, Tokyo) using the Genetyx program (Software Development). The rice kinesin K16 motor domain (K16MD) plasmid was transformed into BL21 (DE3) pLysE for expression in *Escherichia coli*. The K16MD was purified by Ni-NTA metal affinity chromatography according to the method of Shibuya *et al*. (*9*). Purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which revealed a single band on Coomassie-stained gels. Samples were dialyzed against 120 mM NaCl, 30 mM Tris-HCl, pH7.5, 1 mM DTT, and stored at –80°C until use. Tubulin was purified from porcine brain as described by Hackney (*26*).

UV Irradiation—K16MD and Bz_2-εATP containing solutions were placed on ice and irradiated with 302 nm UV light for selected periods of time using a UVM-57 lamp (16 W, Ultra-Violet Products) mounted 2 cm above the surface of the stirred solution of 100–500 μl containing 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$ in the presence or absence of 3 mM nucleotide.

SDS-PAGE—Protein analysis was performed in 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% SDS at a constant voltage (200 V) in the discontinuous buffer system of Laemmli (*27*). Peptide bands were visualized by staining with Coomassie Brilliant Blue. Molecular masses of the peptide bands were estimated by comparing their mobilities with markers of known molecular weight.

ATPase Activity—The ATP assay was performed for 1 μM K16MD in the presence of 2 mM ATP, 50 mM KCl, 10 mM imidazole-HCl, pH 7.0, 3 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis (beta-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 1 mM β-mercaptoethanol in the presence or absence of 5 μM microtubule at 25°C. The reaction was stopped by the addition of 10% trichloroacetic acid, and the released P_i was measured by the method of Youngburg and Youngburg (*28*).

Hydrolytic Cleavage Crosslinks—Hydrolytic cleavage of the crosslinks was performed by the method of Maruta *et al*. (*29*). The cross-linked sample was incubated in alkaline solution (0.5 M $NaHCO_3$, 1% SDS, and 0.1 M NaOH) at 37°C for 24 h to hydrolyze the ester bond in Bz_2-εATP. After dialysis against the running buffer (0.025 M Tris base, pH 8.3, 0.192 M glycine, 0.1% SDS) for SDS-PAGE to remove the excess alkaline reagent, the sample was applied to SDS-PAGE.

RESULTS AND DISCUSSION

We recently succeeded in expressing and characterizing a new rice plant-specific kinesin, K16 (*17*). The motor domain of the kinesin shares 43.2% amino acid sequence homology with mouse brain kinesin, and the tail domain is approximately 20% homologous to that of mouse brain kinesin. We have demonstrated that the rice K16MD unusually retains enzymatic characteristics even after the removal of ADP from the ATPase site, in contrast to conventional kinesins. Therefore, K16MD may have a somewhat different conformation from conventional kinesins. In this study, the fluorescent photoreactive ATP derivative, Bz_2-εATP, was synthesized in order to study the structural characteristics of the novel rice K16MD.

The ATP derivative carries a photoreactive benzoylbenzoyl group at the 3′(2′) position of ribose and the fluorescent 1,N^6-etheno adenine. Bz_2-εATP was hydrolyzed by kinesin as shown in Table 1. However, the activity of the microtubule-dependent ATPase was much lower than that of regular ATP. We estimated the apparent affinity of Bz_2-εATP for the ATPase site of kinesin using the fluorescent ATP analogue 3′(2′)-*O*-{6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-hexznoic}-ATP (NBD-ATP),

Table 1. **Microtubule-dependent ATPase activity of Bz_2-εATP by K16MD.**

Hydrolysis (P_i-mol/K16MD-mol/min)	–MTs	+MTs
Regular ATP	1.62	16.2
Bz_2-εATP	0.26	0.34

1 μM K16MD was preincubated for 5 min in 10 mM imidazole-HCl, pH 7.0, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol and 5 mM microtubule. The ATPase reaction was started by the addition of 1 mM ATP or 1 mM Bz_2-εATP at 25°C.

which has an affinity similar to that of regular ATP (*30*). It is known that NBD-ATP increases its fluorescence when it binds to the ATPase site (*9*). Even 100-fold excess molar of Bz_2-εATP did not inhibit the binding of NBD-ATP to kinesin. Therefore, the affinity of Bz_2-εATP for the kinesin motor domain is much lower than that of regular ATP. Since ATP analogues carry fluorescent probes at the 3′(2′) position of ribose, *e.g.*, Mant-ATP retains an affinity similar to that of regular ATP, it is thought that the 1,N^6-etheno moiety of Bz_2-εATP reduces the affinity to kinesin.

We also performed photo affinity labeling of rice kinesin with Bz_2-εATP. The K16MD was irradiated with UV light at 302 nm in the presence of two-fold excess molar Bz_2-εATP. The bound Bz_2-εATP was analyzed by measuring its fluorescence in the SDS-PAGE gel. As expected from the low affinity of Bz_2-εATP for the ATPase site of the kinesin, Bz_2-εATP was not incorporated into the K16MD (data not shown). On the other hand, in the presence of ATP, a new band at around 100 kDa was produced by the photoreaction of Bz_2-εATP with K16MD (Fig. 1, lane 2). This band was not observed in the absence of ATP (Fig. 1, lane 3). Based on the molecular size of the band, the product was considered to be a dimer of K16MD. It is plausible that the dimer is produced by intermolecular cross-linking mediated by Bz_2-εATP upon photoreaction. To confirm this, we examined the concentration dependency of Bz_2-εATP and UV irradiation time dependency of the production of the 100 kDa band. The formation of the 100 kDa product increased with Bz_2-εATP concentration and reached a maximum value (24%) at an approximately two-fold excess molar Bz_2-εATP for 10 μM K16MD (Fig. 2A). The production of the band also increased with irradiation time, and saturated at about 20 min. In the absence of ATP, the 100 kDa band was not observed (Fig. 2A, inset). ADP also showed a similar effect on the cross-linking reaction (Fig. 2B). The K16MD used in these experiments did not contain ADP in the active site. Interestingly, K16MD retains total enzymatic activity even after the removal of the ADP bound in the active site, in contrast to the well known conventional kinesins (unpublished; our data). The ADP-free kinesin has a conformation that is not cross-linked. ATP or ADP binding to the ATPase site of the rice kinesin is necessary to induce cross-linking. Therefore, it appears that nucleotide binding to the active site induces a conformational change in K16MD and cross-linking mediated by Bz_2-εATP upon photoreaction. Since the ATPase site is occupied by ADP, it is plausible that the cross-linking occurs between sites other than the ATP binding sites. As shown in Fig. 2A, the cross-linking saturated at a two-fold molar excess of Bz_2-εATP for kinesin, the affinity for the second binding site in K16MD is relatively high. The Bz_2-εATP concentration for half maximal cross-linking is approximately 4 μM.

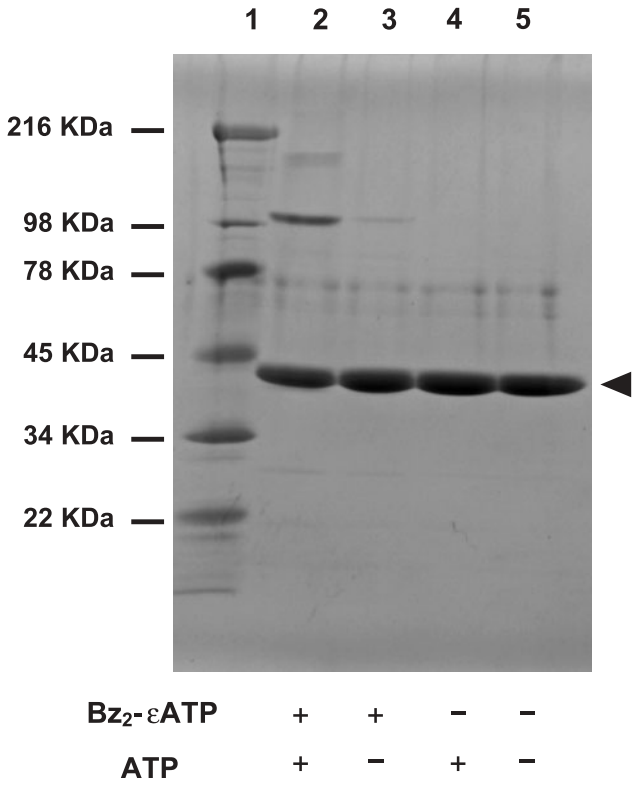

Fig. 1. **SDS-PAGE of K16MD irradiated by UV light in the presence of Bz_2-εATP.** K16MD (10 μM) was cross-linked with 20 μM Bz_2-εATP. Buffer conditions were as follows: 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$ in the presence or absence of 3 mM ATP. The samples were irradiated on ice for 30 min with 366 nm UV light. Lane 1, molecular mass markers, 216, 98, 78, 45, 34 and 22 kDa from top to bottom; lane 2, presence of Bz_2-εATP and regular ATP; lane 3, presence of Bz_2-εATP and absence of regular ATP; lane 4, absence of Bz_2-εATP and presence of regular ATP; lane 5, absence of Bz_2-εATP and regular ATP. Arrowhead indicates the K16MD (40 kDa).

The ATPase activity of K16MD photo reacted with Bz_2-εATP in the presence of ATP decreased with irradiation time (Fig. 3). The plot of the inhibition value corrected for the control reached a maximum value (50%) at around 20 min (Fig. 3, dotted line and cross). Therefore, 50% activity of the K16MD was reduced by photo-modification with Bz_2-εATP. It is known that the photoreactive benzoylbenzoyl group reacts with high efficiency under UV irradiation (*23*, *24*). For skeletal muscle myosin, UV irradiation yields 50–60% covalent incorporation into the ATPase site (*24*). The 50% inhibition of the ATPase activity of K16MD photo-modified by Bz_2-εATP corresponds to that efficiency. However, the maximum value of inhibition is inconsistent with the value of 24% cross-linking. This suggests that approximately half of the photo incorporated Bz_2-εATP did not participate in cross-linking. Probably the other photoreactive moiety in Bz_2-εATP has a lower efficiency than the benzoylbenzoyl group. As shown in Fig. 3, the inhibition of microtubule-dependent ATPase activities corresponds well to that of the basal ATPase activities. Therefore, it is thought that the cross-linked site is a functionally substantial region that affects the catalytic site directly.

Considered together, these results suggest that Bz_2-εATP performs unusually as a bifunctional crosslinker. Since the benzoylbenzoyl group is a well known photoreactive group, Bz_2-εATP can bind to kinesin through the benzoylbenzoyl group. Another possible photoreactive moiety in Bz_2-εATP is the 1,N^6-ethenyl in the base part. To determine whether another photoreactive moiety exists in the base part of Bz_2-εATP, we treated the motor domain cross-linked by Bz_2-εATP with mild alkaline solution to cleave the ester bond that links the benzoylbenzoyl group and

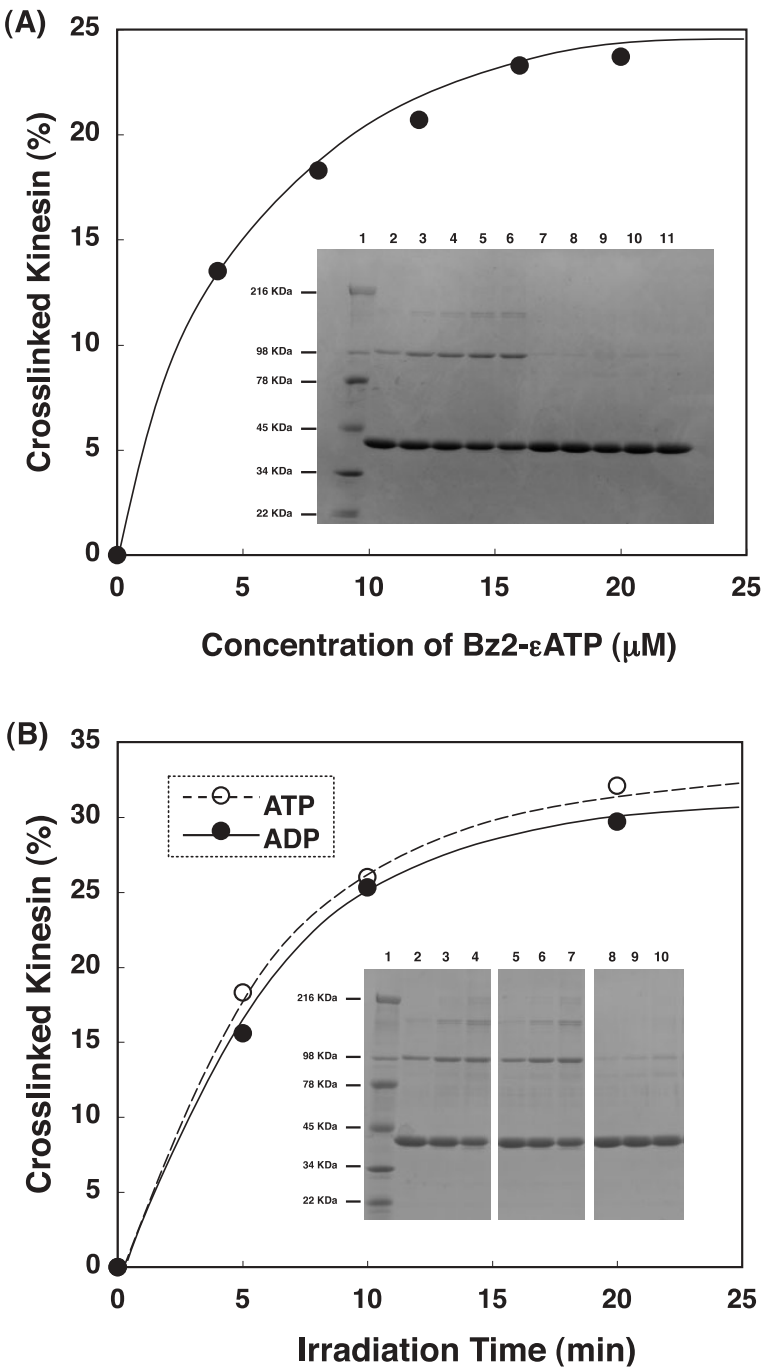

Fig. 2. **(A) Bz_2-εATP concentration dependency of the 100 kDa intermolecular cross-linking product in K16MD.** 10 μM K16MD was incubated with 0–20 μM Bz_2-εATP in 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$ in the presence or absence of 3 mM ATP. The samples were irradiated for 20 min with 302 nm UV light on ice. Rate of cross-linked K16MD with Bz_2-εATP = (The concentration of cross-linked K16MD)/(The total concentration of K16MD) × 100. Band intensities were analyzed with an Image analyzer. (Inset) Coomassie Blue–stained SDS-PAGE gels of intermolecular cross-linking. Lane 1, molecular mass markers, 216, 98, 78, 45, 34 and 22 kDa from top to bottom; lanes 2 and 7, 4 μM Bz_2-εATP; lanes 3 and 8, 8 μM Bz_2-εATP; lanes 4 and 9, 12 μM Bz_2-εATP; lanes 5 and 10, 16 μM Bz_2-εATP; lanes 6 and 11, 20 μM Bz_2-εATP; lanes 2–6, presence of 3 mM ATP; lanes 7–11, absence of nucleotide. **(B) Irradiation time course of 100 kDa band formation in K16MD.** 10 μM K16MD was incubated with 20 μM Bz_2-εATP in 120 mM NaCl, 30 mM Tris-HCl, pH7.5, 3 mM $MgCl_2$ in the presence 3 mM ATP or 3 mM ADP. The samples were irradiated for 0 to 20 min with 302 nm UV light on ice. Rate of cross-linked K16MD with Bz_2-εATP = (The concentration of cross-linked K16MD)/(The total concentration of K16MD) × 100. Band intensities were analyzed with an Image analyzer. (Inset) Coomassie blue-stained SDS-PAGE gels of intermolecular cross-linking. Lane 1, molecular mass markers, 216, 98, 78, 45, 34 and 22 kDa from top to bottom; lanes 2, 3 and 4, presence of ADP; lanes 5, 6 and 7, presence of ATP; lanes 8, 9 and 10, absence of nucleotide. The samples were UV irradiated for 5 min (lanes 2, 5 and 8), 10 min (lanes 3, 6 and 9), or 20 min (lanes 4, 7 and 10) on ice.

ribose of εATP in Bz_2-εATP. We previously demonstrated that ester bonds that link azidonitrobenzoic acid and ribose of 8-N_3-ATP in 3′(2′)-*O*-azidonitrobenzoic-8-N_3-ATP can be readily cleaved by mild alkaline treatment without hydrolysis of peptide bonds (*28*). As shown in Fig. 4 (lane 3), after alkaline hydrolysis of photo cross-linked kinesin, the 100-kDa band was abolished completely and the intensity of the 40-kDa band increased slightly. Moreover, no 1,N^6-ethenoadenosine fluorescence was observed in the cross-linked 100-kDa band. As the ribose and phosphate moieties in ATP are not photoreactive, these results suggest that the 1,N^6-ethenyl in the base part of Bz_2-εATP may be photoactivated by UV irradiation produce crosslinks. Although we cannot yet address the possible photoreactive mechanism of the fluorescent 1,N^6-ethenyl moiety in εATP, which has widely expanded π electron resonance, at this stage, the moiety is most plausible in εATP.

Whether the photo cross-linking reaction by Bz_2-εATP also occurs with other kinesins requires further investigation. The K16MD has unique characteristic properties compared with the conventional kinesin motor domain. For instance, it is known that kinesins from which tightly bound ADP is removed from the ATPase site are, in general, very labile (*31*, *32*). In contrast, rice kinesin K16 retains its enzymatic properties even after the

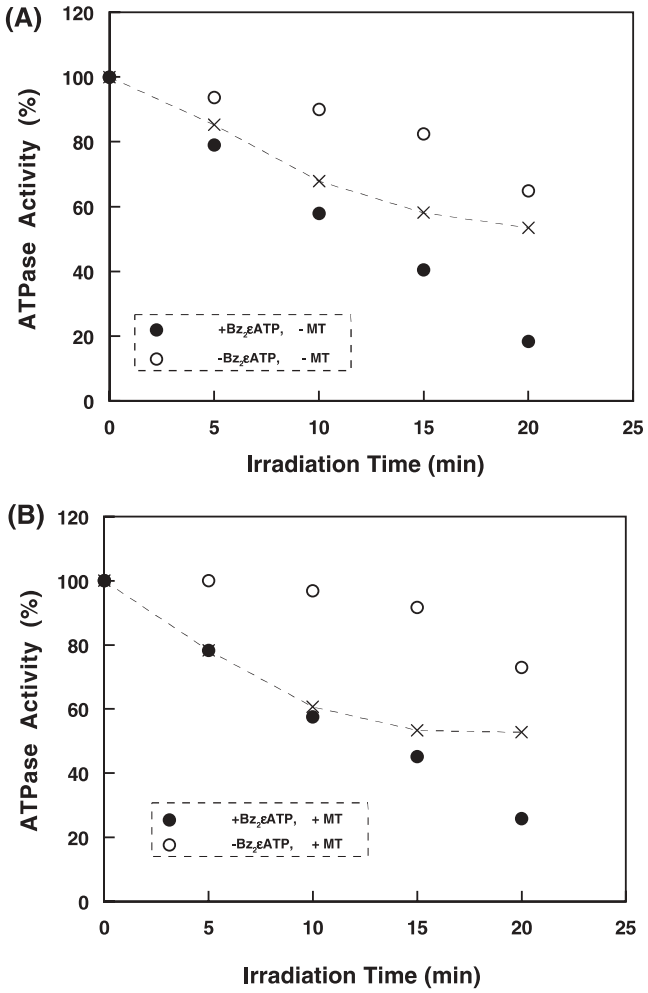

Fig. 3. **Inactivation of K16MD ATPase by photo cross-linking reaction with Bz$_2$-εATP.** 10 μM K16MD was incubated with 20 μM Bz$_2$-εATP in 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$ and 3 mM ATP. The samples were irradiated for 0 to 20 min with 302 nm UV light on ice. The ATP assay was performed using 1 μM K16MD in the presence of 2 mM ATP, 50 mM KCl, 10 mM Imidazole-HCl, pH 7.0, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol in the absence (A) or presence (B) of 5 μM microtubules (MT) at 25°C.

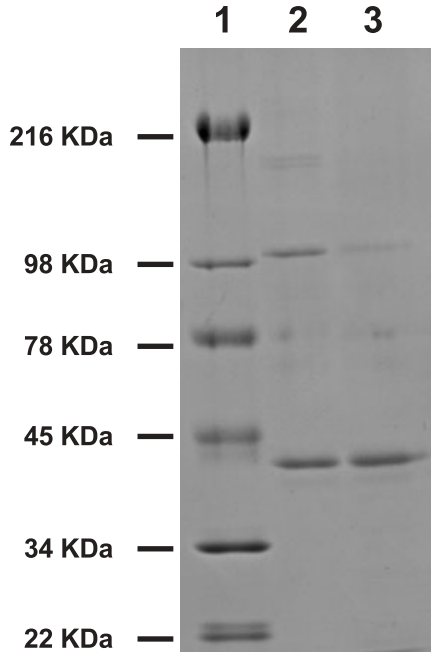

Fig. 4. **Cleavage of cross-linked K16MD with Bz$_2$-εATP by alkaline treatment.** Cross-linked samples were incubated in 0.5 M $NaHCO_3$, 1% SDS, 0.1 M NaOH at 37°C for 24 h, and then dialyzed against the SDS running buffer (0.025 M Tris base, pH 8.3, 0.192 M glycine, 0.1% SDS). The cleaved samples were analyzed by SDS-PAGE in 7.5–20% acrylamide gradient gels. Lane 1, molecular mass markers, 216, 98, 78, 45, 34 and 22 kDa from top to bottom; lane 2, cross-linked K16MD with Bz$_2$-εATP; lane 3, cleavage sample of cross-linked K16MD subjected to alkaline treatment.

removal of ADP from the ATPase site. Therefore, it is expected that the cross-linking reaction by Bz$_2$-εATP may reflect differences between rice kinesin K16 and other kinesins.

Next, mouse brain kinesin was reacted with Bz$_2$-εATP. No new crosslinked-band of the mouse kinesin motor domain was observed. On the other hand, for the 560–amino acids mouse kinesin with a coiled coil tail region that forms dimers, a new cross-linked band was observed. This result strongly suggests that the K16MD forms dimers in the presence of nucleotides. We also performed the photo cross-linking reaction by Bz$_2$-εATP on the motor domain of skeletal muscle myosin, which has an extremely conserved motor domain with kinesin. Neither the subfragment-1 (monomer) nor the heavy mero myosin (dimer) was cross-linked by Bz$_2$-εATP. Therefore, the photo cross-linking by Bz$_2$-εATP is a specific reaction for kinesin.

In conclusion, we have demonstrated in the present study that a fluorescent photoreactive ATP derivative that is generally used for photoaffinity labeling of ATP binding sites, unusually acts as a bifunctional crosslinker. We also showed that the K16MD changes its conformation significantly in the presence of nucleotides, resulting in the formation of dimers and cross-linking by Bz$_2$-εATP upon UV irradiation. Moreover, the motor domain of rice kinesin K16 has unique properties in contrast to those of conventional kinesins. The novel photo-crosslinking reaction with Bz$_2$-εATP is applicable especially to the study of conformational changes in kinesin.

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