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Interaction of a New Fluorescent ATP Analogue with Skeletal Muscle Myosin Subfragment-1[1]

Shinsaku Maruta,*[2] Yoshiaki Mizukura,* and Shigeru Chaen†

Department of Bioengineering, Faculty of Engineering, Soka University, Hachioji, Tokyo 192-8577; and †Department of Molecular Physiology, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613

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A new fluorescent ribose-modified ATP analogue, 2′(3′)-*O*-{6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic}-ATP (NBD-ATP), was synthesized and its interaction with skeletal muscle myosin subfragment-1 (S-1) was studied. NBD-ATP was hydrolysed by S-1 at a rate and with divalent cation-dependence similar to those in the case of regular ATP. Skeletal HMM supported actin translocation using NBD-ATP and the velocity was slightly higher than that in the case of regular ATP. The addition of S1 to NBD-ATP resulted in quenching of NBD fluorescence. Recovery of the fluorescence intensity was noted after complete hydrolysis of NBD-ATP to NBD-ADP. The quenching of NBD-ATP fluorescence was accompanied by enhancement of intrinsic tryptophan fluorescence. These results suggested that the quenching of NBD-ATP fluorescence reflected the formation of transient states of ATPase. The formation of S-1·NBD-ADP·BeF_n and S-1·NBD-ADP·AlF_4^- complexes was monitored by following changes in NBD fluorescence. The time-course of the formation fitted an exponential profile yielding rate constants of 7.38 × 10^{-2} s^{-1} for BeF_n and 1.1 × 10^{-3} s^{-1} for AlF_4^-. These values were similar to those estimated from the intrinsic fluorescence enhancement of trp due to the formation of S-1·ADP·BeF_n or AlF_4^- reported previously by our group. Our novel ATP analogue seems to be applicable to kinetic studies on myosin.

Key words: ATPase, energy transduction, fluorescent labeling, kinetics, transient state analogue.

Kinetic studies on the actomyosin ATPase cycle have established the kinetic pathway of actomyosin ATPase. ATP is hydrolysed by myosin through 5 steps in the ATPase cycle. These steps could be observed by measuring intrinsic tryptophan fluorescence, which reflects the conformation of intermediate compounds. However, it is not clear at present how the myosin head changes its conformation in the ATPase cycle, a process directly related to energy transduction. Since the intermediates in the ATPase cycle only have a limited lifetime, identification of stable analogues corresponding to these intermediates would be useful.

Goodno (*1*) was the first to demonstrate that in the presence of MgADP, orthovanadate (V_i) could form a stable ternary complex with myosin corresponding to a 1:1:1 ternary complex designated as M·ADP·V_i, which resembles the steady-state intermediate M**·ADP·P_i of the ATPase cycle (*1*). More recently, it was shown that the phosphate analogues of various fluorometals, aluminium fluoride (AlF_4^-) (*2, 3*), beryllium fluoride (BeFn) (*2–4*), scandium fluoride (ScFn) (*5*), gallium fluoride (GaFn) (*6*), and magnesium fluoride (MgFn) (*7, 8*), also form stable myosin·ADP·fluorometal ternary complexes. Differences in the interaction between actin and each of the complexes may mimic distinct reaction intermediates of the myosin ATPase cycle (*2*). Moreover, recent crystallographic studies have revealed differences between the ternary complexes of AlF_4^-, BeFn, and V_i, with respect to the conformation of the C-terminal segment of the truncated myosin head from *Dictyostelium discoideum* myosin II (S-1Dc) (*9, 10*). In our studies, we proposed that the possible alignment of the ternary complexes in the ATPase cycle is as follows:

$$\mathrm{M^{*}{\cdot}ATP \rightarrow M^{**}{\cdot}ADP{\cdot}P_i \rightarrow M^{*}{\cdot}ADP}$$

(*BeFn, **BeFn, MgFn, ScFn, AlF4)

Where *BeF_n and **BeF_n indicate different species of MgF_n depending on the number of F that binds to Be.

It is possible to determine the sequential changes in conformation in the ATPase cycle, which may reflect energy transduction, by comparing the structures of the ternary complexes. In a series of recent studies, we analysed the global conformation of the ternary complexes in solution by small-angle synchrotron X-ray scattering and showed that the complexes became compact or round in shape (*6, 7*). In another study, we also analysed the local conformation at the highly reactive cystein residues of SH1 (707) and SH2 (697), and lysine (*83*) by means of chemical modifications,

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[2]To whom correspondence should be addressed. Phone: +81-426-91-9443, Fax: +81-426-91-9312, E-mail: shinsaku@t.soka.ac.jp

Abbreviations: AlF_4^-, aluminium fluoride; BeFn, beryllium fluoride; *DTT, dithiothreitol*; FEDA-ATP, 2′(3′)-*O*-[*N*-[2-[3-(5-fluoresceinyl)-thioureido]ethyl]carbamoyl]adenosine 5′-triphosphate; Mant-ATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)-ATP; NBD-ATP, 2′(3′)-*O*-{6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic}-ATP; REDA-ATP, 2′(3′)-*O*-[*N*-[2-[3-[5(6)-tetraethylrhodamine]-thioureido]ethyl] carbamoyl]adenosine 5′-triphosphate; S1, myosin subfragment-1; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

and our results suggested that AlF_4^- and ScF_n complexes could be distinguished from other complexes (*11*).

The introduction of fluorescent ATP derivatives that behave with myosin in a manner similar to that of ATP and have fluorescence signals that allow the monitoring of nucleotide binding could greatly contribute to the study of the myosin·ADP·fluorometal ternary complexes. Several types of fluorescent ATP analogues have already been synthesized, *e.g.*, Mant-ATP (*12, 13*), 1-N^6-ethenoadenosine triphosphate (*14*), and 1-N^6-etheno-2-aza-ATP (*15–18*), and used in kinetic studies on myosin. Furthermore, fluorescent ATP analogues are useful not only for the analysis of ATP-utilizing enzymatic reactions but also for structural studies involving the fluorescence polarization and energy transfer techniques.

In the present paper, we report the synthesis of a novel fluorescent-labelled ATP analogue, NBD-ATP, to monitor the formation of ternary complexes and to analyse localized conformation of the complexes in detail. Our results showed that the affinity of NBD-ATP for myosin was similar to that of ATP, and that the binding constant of NBD-ADP as to S-1 is equal to the corresponding values in the case of ADP. The formation of the myosin·NBD-ADP·fluorometal ternary complexes could be monitored as changes in the fluorescence of NBD-ADP. Our results indicate that our novel ATP analogue could be applied for the study of ternary complexes.

EXPERIMENTAL

Synthesis of 2′(3′)-O-{6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic}-ATP (NBD-ATP)—Coupling of ATP and 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid (NBD acid) was carried out by the method of Guillory and Jeng (*19*). NBD acid (68 μmol) and carbonyldiimidazole (204 μmol) were stirred for 30 min at room temperature in 0.48 ml of dry DMF. ATP (34 μmol) was dissolved in 0.51 ml of water and then added dropwise to the reaction mixture. The coupling reaction was allowed to proceed for 48 h at room temperature under continuous stirring. The reaction mixture was evaporated to dryness and the residue dissolved in 1 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 as to the absorbance at 275 nm. Three major peaks were obtained. The first peak eluted was of unreacted ATP, and third was of the unreacted NBD acid. The second peak contained NBD-ATP. The fractions were collected and lyophilized. The purity of the product was analysed by TLC on silica gel plates (Silica Gel-70 F254; Wako Pure Chemical, Osaka) using 1-butanol/acetic acid/H_2O (5:1:3, by volume) as the developing solvent, and the R_f value was 0.35.

Preparation of Protein—Myosin was prepared from chicken breast muscle as described by Perry (*20*), and was digested with α-chymotrypsin to obtain subfragment-1 (S-1) as reported by Weeds and Taylor (*21*).

Fluorescence Measurement—Fluorescence was measured at 25°C with an F-2500 spectrofluorometer (Hitachi, Tokyo).

Measurement of the Sliding Velocity of F-Actin on Myosin Using NBD-ATP—Skeletal muscle myosin heavy mero myosin (80 μg/ml) in a high salt solution (0.5 M KCl, 20 mM PIPES, pH 7.5, 2 mM $MgCl_2$, and 0.1 mM DTT) was perfused into a flow cell made from a nitro-cellulose–coated glass coverslip. After 5 min, the flow cell was rinsed with a high salt solution including 1 mg/ml BSA and then left for 5 min, and finally 10 nM F-actin filaments labelled with rhodamine-conjugated phalloidin (Molecular Probe, Eugene, OR) in a motility buffer (25 mM KCl, 25 mM imidazole, pH 7.5, 0.1 mM ATP, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT, 1 mg/ml BSA, 0.2% methyl cellulose, 1% β-mercaptoethanol, 4.5 mg/ml glucose, 210 μg/ml glucose oxidase, and 35 μg/ml catalaser) were perfused into the flow cell. The above treatments were performed at 0°C. The flow cell was then equilibrated for 5 min between the temperature-controlled slide carrier and temperature-jacketed objective of an inverted-microscope. Methylcellulose was required to observe the F-actin sliding on both isoform preparations. Fluorescent F-actin filaments were examined under an inverted microscope [Axiovert 35; Zeiss Neofluoar × 100 objective (N.A. 1.3)] and SIT camera (C2400-08; Hamamatsu Photonics, Hamamatsu), and recorded on S-VHS videotape (AG7355; Panasonic, Osaka). Selected video frames were digitised with a frame grabber (LG-3; Scion, Frederick, MD) and NIH image analysis software. The sliding velocity of fluorescent F-actin was calculated by measuring the displacement of a filament between successive video frames using a program for tracking within NIH image analysis software.

RESULTS

Synthesis and Characterization of NBD-ATP—Many ATP-requiring enzymes, including myosin, tolerate chemical modifications at the ribose ring of the ATP molecule. Therefore, we designed a fluorescent ATP analogue, 2′(3′)-*O*-{6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic} ATP (NBD-ATP) (Fig. 1A), which carried a fluorescent probe at the ribose. The reason for selecting the NBD group is that the nitrobenzoxadiazole (NBD) fluorophore is highly environment-sensitive. NBD-ATP was synthesized by coupling of ATP and 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanoic acid (NBD acid) according to the established method (*19*). The products were purified by HPLC on an RP-C18 column as shown in Fig. 1B. The first and third major peaks were identified as unreacted ATP and NBD acid, respectively, on TLC on silica gel plates. The second peak eluted at 52–58 min was found to be NBD-ATP for the following reasons. (i) The R_f value on TLC was different from those of ATP and NBD acid. (ii) The NO_2 group was identified by IR spectrum analysis. (iii) The UV spectrum showed peaks of ATP at 260 nm and NBD at 485 nm (Fig. 2A), and (iv) mass spectral analysis of the product revealed a mass identical to that of NBD-ATP.

The fluorescence spectrum of NBD-ATP in buffer (pH 7.5) showed an excitation maximum at 480 nm and an emission maximum at 535 nm (Fig. 2B), which were shifted by about 10–20 nm to longer wavelengths compared with the spectrum of free NBD acid in methanol. No obvious contamination was observed on TLC analysis, yet myosin hydrolysed this compound, resulting in the release of inorganic phosphate. Table I summarizes the divalent cation dependence of the hydrolysis rate of NBD-ATP. It is well known that myosin ATPase activity depends on the pres-

ence of divalent cations. Regular ATP exhibits a very slow hydrolysis rate in the presence of Mg^{2+}, which is markedly accelerated by actin, a fast Ca^{2+}-ATPase hydrolysis rate and a very fast EDTA (K^+)-ATPase hydrolysis rate. The divalent cation dependency of NBD-ATP as to its ATPase activity was similar to that of regular ATP, although EDTA (K^+)-ATPase is about three times slower.

To determine whether or not NBD-ATP could support chemomechanical transduction in the actomyosin system, a standard *in vitro* motility assay was used to examine the ability of this analogue to support actin sliding over immobilized myosin. Skeletal muscle myosin HMM supported actin translocation with NBD-ATP as substrate at a slightly higher velocity than in the case of regular ATP (Fig. 3). In the presence of 10 μM nucleotide, the velocities for NBD-ATP and ATP were 0.54 and 0.76 μm/s, respectively. This indicates that the chemomechanical efficiency of NBD-ATP for the actomyosin motility system is almost the same as that of ATP.

Fluorescent Studies on the Interaction of NBD-ATP with S1—Under single-turnover conditions, the addition of S1 to NBD-ATP resulted in an immediate decrease of 40% in

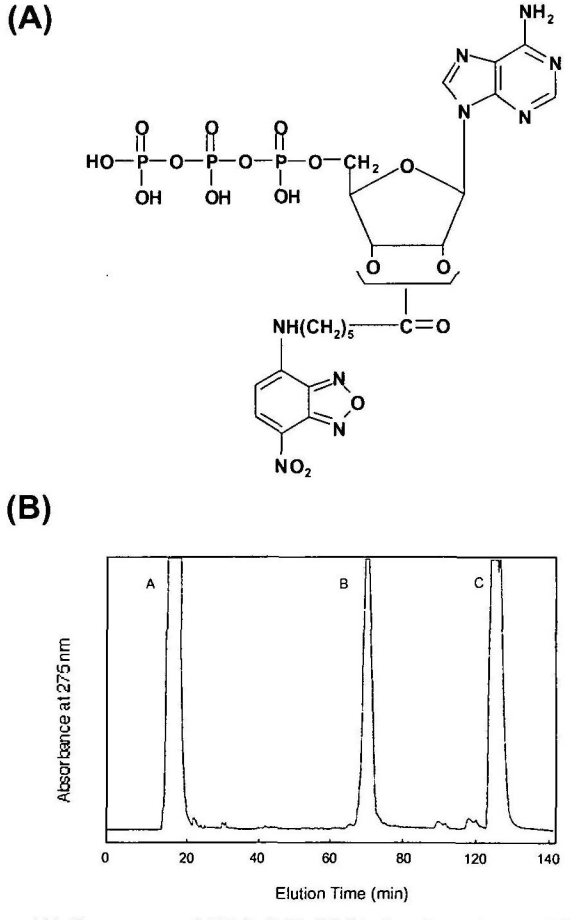

Fig. 1. **(A) Structure of 2′(3′)-*O*-{6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic}-ATP (NBD-ATP). (B) Purification of NBD-ATP by reverse phase column chromatography.** The sample in water was applied on a Shodex RP-18 column equilibrated with 0.1% TFA. Fractions were eluted with a linear acetonitrile gradient of 0–90% over 130 min at a flow rate of 1 ml/min. Peak A, unreacted ATP; peak B, NBD-ATP; peak C, unreacted NBD acid.

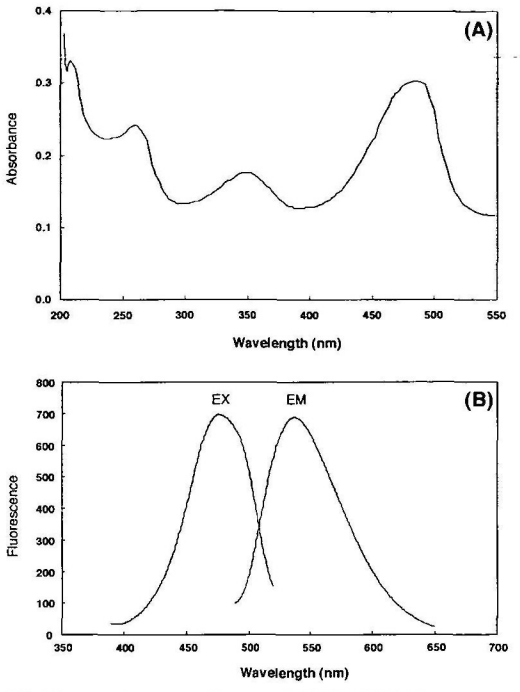

Fig. 2. **(A) Absorption spectrum of NBD-ATP.** The spectrum was measured in 20 mM Tris-HCl, pH 7.5, using a Shimadzu UV-2200 spectrophotometer. **(B) Fluorescence excitation and emission spectra of NBD-ATP.** Spectra were measured in 20 mM Tris-HCl, pH 7.5, at 25°C using a HITACHI RF-2500 fluorescence spectrophotometer. The excitation and emission wavelengths were 475 and 535 nm, respectively.

TABLE I. **Bivalent cation-dependence of ATPase activity.** The incubation mixture contained 1 mM S-1, 0.5 mM ATP (or ATP analogue), 0.5 M KCl, and 30 mM Tris-HCl, pH 7.5, at 25°C. (a) 5 mM $MgCl_2$; (b) 5 mM $CaCl_2$; (c) 5 mM EDTA.

	ATP	NBD-ATP
	(mol of P_i/mol of S-1·min)	
(a) Mg^{2+}	0.4	0.4
(b) Ca^{2+}	22.0	23.5
(c) EDTA(K^+)	128.7	47.4

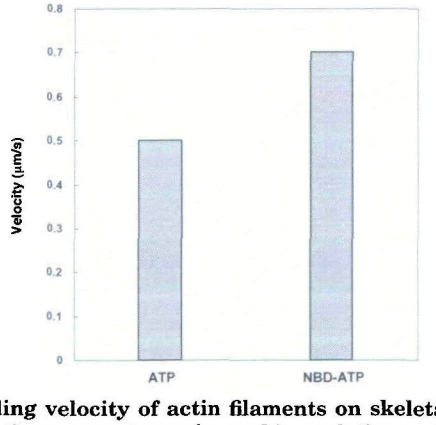

Fig. 3. **Sliding velocity of actin filaments on skeletal muscle myosin.** Actin movement was observed in a solution containing 0.5 M KCl, 20 mM PIPES, pH 7.0, 2 mM $MgCl_2$, and 0.1 mM DTT. Rabbit skeletal muscle HMM (80 μg/ml) was perfused into a flow cell made from a nitrocellulose-coated glass coverslip.

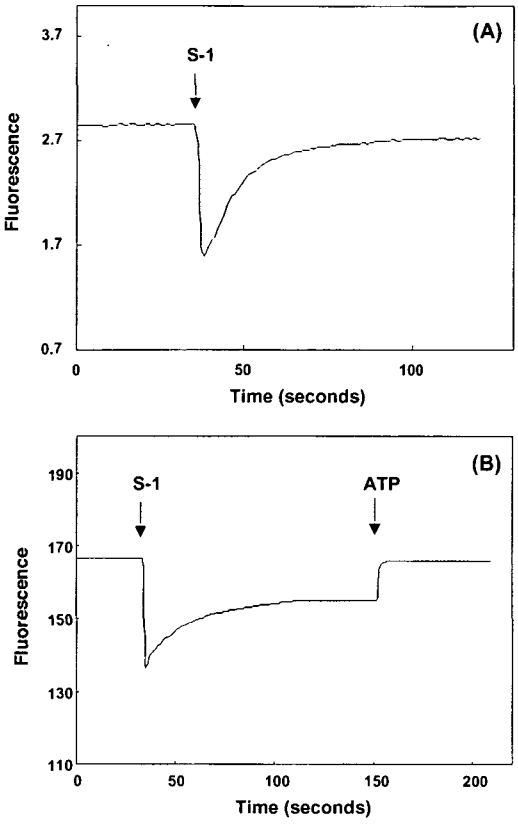

Fig. 4. **(A) Fluorescence transients observed upon addition of NBD-ATP to S1 under single-turnover conditions.** The buffer conditions were as follows: 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ at 25°C. 5 μM S-1 was mixed with 1 μM NBD-ATP. **(B) Recovery of NBD-ATP fluorescence upon addition of ATP.** To a 30 μM NBD-ATP solution containing 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ were added 3 μM S-1 and then 0.1 mM ATP. Changes in NBD-ATP fluorescence were measured at 535 nm (excitation at 475 nm).

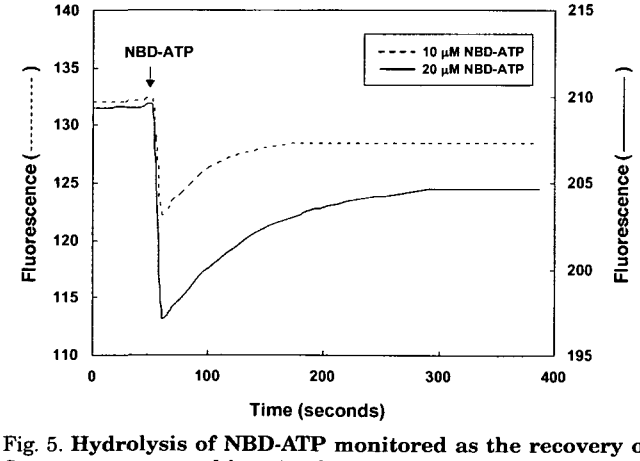

Fig. 5. **Hydrolysis of NBD-ATP monitored as the recovery of fluorescence quenching.** A solution containing NBD-ATP (solid line, 20 μM NBD-ATP; dashed line, 10 μM NBD-ATP) was preincubated at 25°C in a buffer solution of 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$, and the reaction commenced by rapid mixing with 2 μM S-1. The excitation and emission wavelengths were 475 and 535 nm, respectively.

NBD fluorescence but the latter recovered immediately at a rate constant of 0.06 s^{-1}, resulting in hydrolysis of NBD-ATP to NBD-ADP, which is a similar value to the k_{cat} of regular ATP reported by Conibear *et al.* (*17*) (Fig. 4A). Under multiple-turnover conditions, the NBD fluorescence remained at the quenched level during the steady-state but recovered after complete hydrolysis of NBD-ATP. In the presence of 3 μM S^{-1} and 30 μM NBD-ATP, the fluorescence increased by 12% and the addition of excess ATP resulted in complete recovery of the fluorescence indicating that a significant amount of NBD-ADP remained bound to the S1 (Fig. 4B). The addition of 20 μM NBD-ATP to 2 μM S-1 doubled the time for recovery of the fluorescence compared to that of 10 μM NBD-ATP (Fig. 5, dotted line), sug-

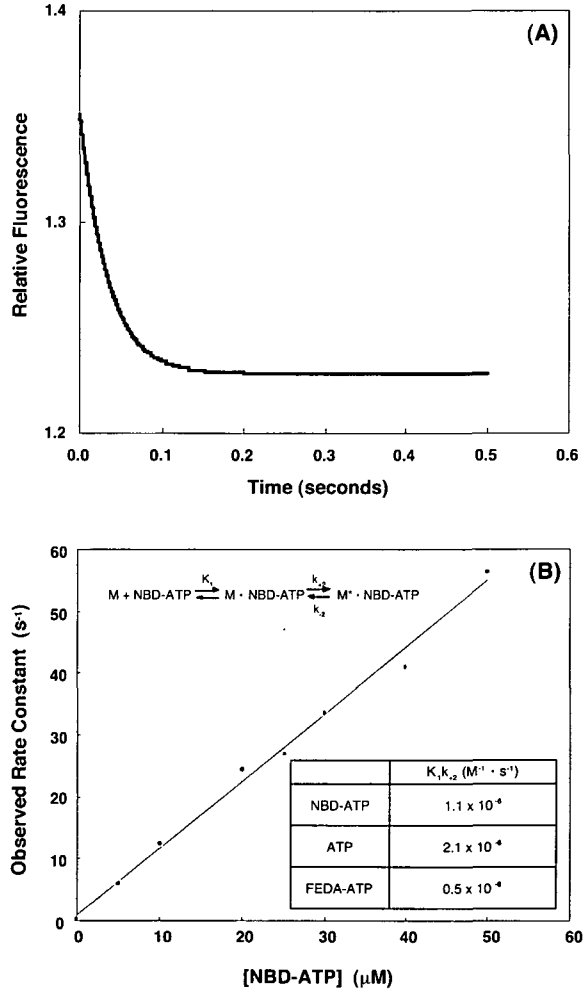

Fig. 6. **(A) Fluorescence transients observed upon addition of excess NBD-ATP (30 μM) to S1 (3 μM) in the stopped-flow apparatus.** The buffer conditions were as follows: 40 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ at 25°C. Zero time is the time at which the flow stopped. The superimposed lines are least-mean-squares fits to a single exponential curve. The reaction was monitored as to NBD fluorescence; k = 33.5 s^{-1} with fluorescence quenching of 8.4%. **(B) Dependence of the fitted rate constants on the concentration of NBD-ATP.** The apparent second-order rate constant, given as the slope of these plots, is 1.1 × 10^{-6} M^{-1} s^{-1} for NBD-ATP. [NBD-ATP]:[S1] = 10 for all data points. The inset shows comparison of the apparent second-order rate constants for NBD-ATP, ATP, and FEDA-ATP.

gesting that quenching of the fluorescence reflects hydrolysis of NBD-ATP. Moreover, the trp fluorescence change was also well accompanied by a change in the fluorescence of NBD-ATP during hydrolysis (data not shown). These results indicate that it is possible to monitor the ATPase reaction by monitoring changes in the fluorescence of NBD-ATP.

To determine the kinetics of ATPase, we studied the first binding of NBD-ATP to S-1 under pseudo-first-order conditions with [NBD-ATP] > [S-1] using stopped flow. The nucleotide to protein ratio was kept at 10 as a compromise between achieving strictly first-order kinetics and maintaining a measurable fluorescence change relative to the background signal. NBD-ATP binding resulted in quenching of NBD fluorescence (Fig. 6A). The time course of quenching of NBD fluorescence was identical in successive pushes of the stopped-flow apparatus. The data fitted a single exponential curve. The apparent secondorder rate constant (1.1×10^{-6}), given as the initial slope of these plots (Fig. 6B), was twofold lower than that for ATP (2.1×10^{-6}). However, it was 2-fold higher than that of FEDA-ATP reported by Conibear *et al.* (*17*).

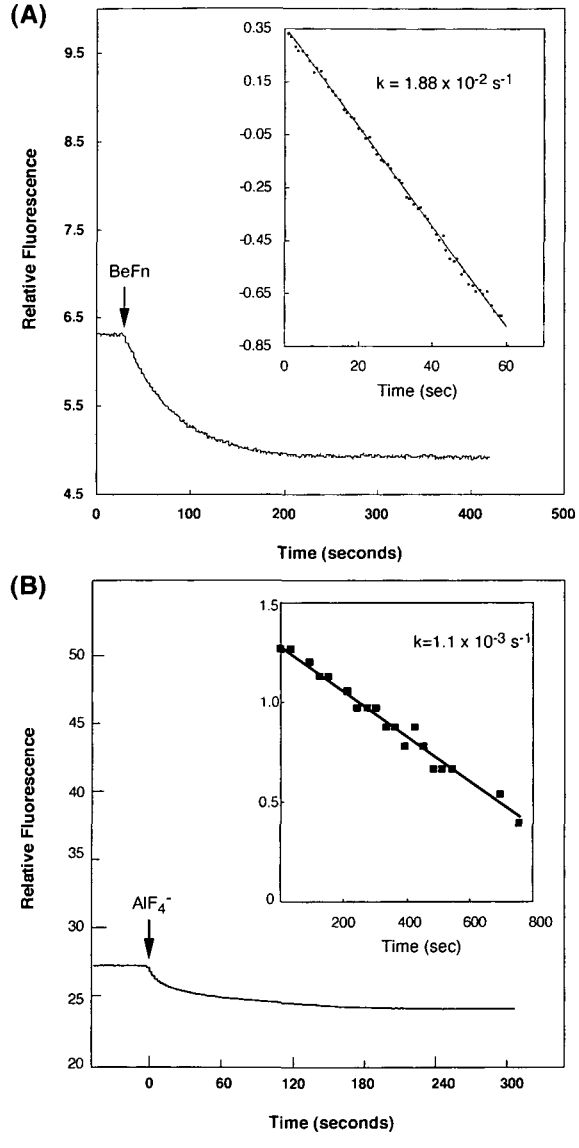

Fig. 7. **(A) Formation of ternary complexes between S-1·NBD-ADP and BeF_n.** NBD-ATP (8 μM) was initially mixed with S-1 (4 μM) in 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ at 25°C. After completion of the hydrolysis, ternary complex formation was initiated by the addition of 0.5 mM BeF_n and monitored at 535 nm with excitation at 475 nm. The inset shows a semilog plot of the BeF_n-induced fluorescence change against time: $k = 1.88 \times 10^{-2}$ s^{-1}. **(B) Formation of a ternary complex between S-1·NBD-ADP and AlF_4^-.** NBD-ATP (8 μM) was initially mixed with S-1 (4 μM) in 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, 2 mM and $MgCl_2$ at 25°C. After completion of the hydrolysis, formation of the ternary complex was initiated by the addition of 0.5 mMAlF_4^- and monitored at 535 nm with excitation at 475 nm. The inset shows a semilog plot of the AlF_4^- induced fluorescence change against time: $k = 1.88 \times 10^{-2}$ s^{-1}.

Formation of S1·NBD-ADP·Fluorometal Ternary Complexes—We have previously demonstrated that in the presence of Mg^{2+}-ADP, myosin forms stable ternary complexes that mimic transient states of ATP hydrolysis with phosphate analogues of AlF_4^- and BeF_n (*2*). Formation of the S1·NBD-ADP·AlF_4^- and S1·NBD-ADP·BeF_n complexes was monitored as the fluorescence change of NBD-ADP (Fig. 7, A and B). After complete hydrolysis of 8 μM NBD-ATP to NBD-ADP by 4 μM S1, the addition of 1 mM BeF_n resulted in a decrease in the fluorescence of NBD-ADP to a level similar to that in the case of steady-state hydrolysis of NBD-ATP accompanied by the formation of a ternary complex. The time-course of the fluorescence change fitted an exponential profile yielding rate constants of 1.88×10^{-2} s^{-1} for BeF_n and 1.1×10^{-3} s^{-1} for AlF_4^-. These values are several times lower than the corresponding values for the trapping of ADP under similar conditions we determined from intrinsic tryptophan fluorescence (*2*).

Finally, we measured fluorescence spectra of S1·NBD-ADP·AlF_4^- and S1·NBD-ADP·BeF_n ternary complexes (Fig.

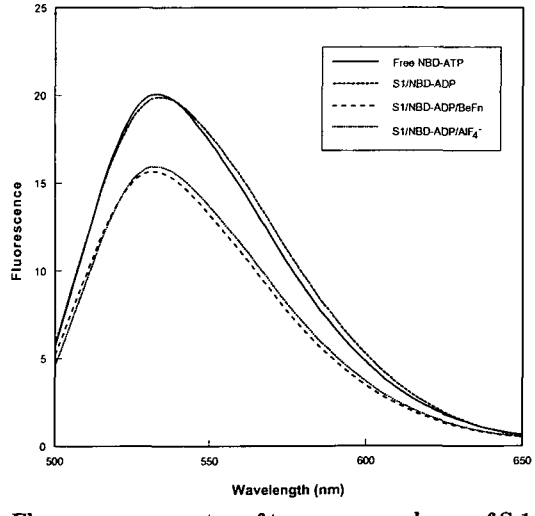

Fig. 8. **Fluorescence spectra of ternary complexes of S-1·NBD-ADP·MgF_n.** The fluorescence spectrum of NBD-ATP (2 μM) free in solution is compared with those of NBD-labeled nucleotides/analogues bound to S-1 (4 μM). NBD-ATP (2 μM) was mixed with S-1 (4 μM) in 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ at 25°C. After completion of the hydrolysis, formation of the ternary complex was initiated by the addition of 0.5 mM BeF_n or 0.5 mM AlF_4^-, followed by incubation for 3 h. NBD-labeled nucleotides were excited at 475 nm, and the emission spectra were measured at 490–660 nm.

8). The fluorescent emission maximum of the ternary complexes was slightly blue shifted from that of free NBD-ADP. However, there were no significant differences between the two ternary complexes, indicating that the conformations at the ATPase site of the two complexes are almost identical.

DISCUSSION

In the present study, we synthesized NBD-ATP, a novel fluorescent ATP analogue, and applied it for the study of myosin·ADP·fluorometal ternary complexes, which mimic the transient state in ATPase. NBD-ATP was readily synthesized by the established methods of coupling ATP and NBD-hexanoic acid (*19*). The fluorescent change of the NBD group in the ATP analogue sensitively reflects the different transient states in the ATPase cycle. Since the NBD fluorophore exhibits an excitation maximum at 475 nm and an emission maximum at 535 nm, the spectrum ranges of NBD overlap well with those of other fluorescent dyes, *e.g.*, Mant (*22*) and Acrylodan (*23*), which are generally used in energy transfer experiments. Our preliminary experiments demonstrated efficient energy transfer between NBD-ADP trapped within the ATPase site by fluorometals and acrylodan fluorophore at SH1 (Cys 707) (unpublished data), suggesting its application for topological studies on myosin.

Several ATP analogues that carry a fluorescent dye similar to NBD-ATP at the ribose moiety have been synthesized and used for kinetic studies on myosin (*12, 13, 17, 18*). This is based on the fact that many ATP-requiring enzymes, including myosin, tolerate chemical modifications at the ribose ring of the ATP molecule. These analogues are present in solution as a mixture of 2′- and 3′-isomers. Several previous studies have demonstrated that there are some differences in the properties of kinetics and fluorescence between the two isomers of the analogues (*24, 25, 26*). Therefore, the 2′- and 3′-isomers of NBD-ATP may also show differences.

As seen for NBD-ATP, the fluorescence of some of these analogues decreases following binding to the active site of myosin. For example, the fluorescence of FEDA-ATP decreased by 28% relative to that of FEDA-ATP when it bound to myosin. At recovery, such a decrease was only 13%. On the other hand, the fluorescence of REDA-ATP decreased by 43% and recovered to 28% (*17*). Hiratsuka (*27*) also reported that binding of Pyr-ADP to S-1 resulted in fluorescence quenching of about 70%. In contrast, the fluorescence of Mant-ATP (*13*) and Cy3-EDA-ATP (*17*) increased following binding to the active site of S-1. Several mechanisms could explain the quenching or enhancement of the fluorescence of ATP analogues. Hydrophobicity around the probes, and hydrogen bonding between probes and amino acid residues are possible factors that could affect the fluorescence intensity. The effects of such factors depend on the conformational changes at the site of binding of the probe used in the study. Therefore, changes in fluorescence intensity reflect the localised conformational changes at the site labeled by the probes.

For NBD-ATP, fluorescence was quenched by 40% during ATP hydrolysis by myosin (Fig. 4A). On the other hand, the fluorescence of NBD-ATP was enhanced by about three times following binding to kinesin, which has a motor domain similar to that of myosin (unpublished data). Recent crystallographic studies on kinesin and myosin have shown differences in several loops near the entrance of the nucleotide pocket (*28, 29*). Kinesin lacks certain loops that are present in the nucleotide binding cleft of myosin. NBD-ATP may sense the loops in myosin that decrease the fluorescence of the fluorophore. In contrast, the fluorophore of Mant-ATP increases its fluorescence when it binds to the site of both kinesin and myosin (*13, 30*).

The NBD fluorophore is highly environment-sensitive. Although it is moderately fluorescent in aprotic solvents, it is almost completely nonfluorescent in aqueous solvents. It has been demonstrated that the NBD fluorophore is moderately polar, and that both its homologous 6-carbon and 12 carbon fatty acid analogues, and the phospholipids derived from these probes tend to sense the lipid-water interface region of membranes instead of the hydrophobic interior (*31*). Therefore, NBD-ATP may also sense the hydrophobic region-water interface near the ATPase site.

Kinetic studies demonstrated that NBD-ATP behaves as a substrate for myosin in a manner similar to regular ATP. The apparent second-order rate constant of binding of NBD-ATP to S-1 is two-fold lower than that for ATP and two-fold higher than that for FEDA-ATP reported by Conibear *et al.* (*17*). Moreover, in the *in vitro* motility assay, NBD-ATP caused actin sliding over immobilized myosin, the velocity of which was almost identical to that for the standard and similar to that observed with regular ATP.

The formation of the ternary complexes of S-1·ADP·AlF_4^- and S-1·ADP·BeF_n was monitored as the fluorescence changes of NBD-ADP. The rate constants estimated from the time-course that fitted an exponential profile were similar to those for trapping ADP, which we were previously estimated from intrinsic trp fluorescence enhancement (*2*). Accordingly, NBD-ADP is a suitable reporter for monitoring the formation of ternary complexes, which mimic the transient state in the ATPase cycle. However, our results did not reveal significant differences in the fluorescence spectra of S-1·NBD-ADP·AlF_4^- and S-1·NBD-ADP·BeF_n. This suggests that the conformation of the ATP binding cleft does not change significantly along the ATP kinetic pathway.

In conclusion, our novel fluorescent ATP analogue, NBD-ATP, is a suitable substrate for myosin and is hydrolysed by myosin in a manner similar to regular ATP. The formation of myosin·ADP·fluorometal ternary complexes could be monitored as the fluorescence change of NBD-ADP. Our results suggest that NBD-ATP could be used in further studies on transient state analogues in the ATPase cycle. Moreover, NBD-ATP may be useful for other nucleotide-requiring proteins.

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