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Synthesis of a novel fluorescent non-nucleotide ATP analogue and its interaction with myosin ATPase

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A novel non-nucleotide fluorescent ATP analogue, *N*-methylanthraniloylamideethyl triphosphate (MANTTP), was designed and synthesized for kinetic studies with ATPases. The interaction of MANTTP with myosin ATPase was characterized. MANTTP was used as a substrate of myosin ATPase, and acceleration of actin-dependent hydrolysis was observed. The fluorescence property of MANTTP was not greatly affected by its binding to the ATPase site of myosin. In contrast, during MANTTP hydrolysis, significant fluorescence resonance energy transfer (FRET) was observed between MANTTP and intrinsic tryptophan residues in the myosin motor domain. Binding of MANTTP and formation of a ternary complex with a myosin- *N*-methylanthraniloylamideethyl diphosphate (MANTDP)-Pi analogue, which may mimic ATPase transient states, were monitored by FRET. The kinetic parameters of MANTTP binding to myosin and MANTDP release from the ATPase site were determined using a stopped-flow apparatus and compared with those of other ATP analogues. This novel fluorescent ATP analogue was shown to be applicable for kinetic analysis of ATPases.

Keywords: ATP analogue/fluorescence/FRET/myosin/kinetics.

Abbreviations: DEAE, diethylaminoethane; DEDA-ATP, 2′(3′)-*O*-[*N*-[2-[[[5-(dimethylamino)-naphthyl]sulphonyl]amino]ethyl]carbamoyl]adenosine 5′-triphosphate; FAB-MS, fast atom bombardment-mass spectrometry; FRET, fluorescence resonance energy transfer; IR, infrared; MANTDP, *N*-methylanthraniloylamideethyl diphosphate; MANTTP, *N*-methylanthraniloylamideethyl triphosphate; myosin, skeletal muscle myosin II; S1, subfragment-1; TLC, thin-layer chromatography.

Several types of ATP analogues with various probes have been synthesized and used for studies of ATPases. The utilized probes and the modification sites on the ATP analogues were designed for various objectives.

Photocrosslinkable ATP analogues were utilized for photoaffinity labeling of ATPase sites. Further, fluorescently labelled ATP analogues were used for kinetic studies of ATPases, *e.g.* myosin and kinesin. Fluorophores were often incorporated into ATP analogues via the hydroxyl group of ribose (*1*–*6*), as done for Mant-ATP (*1*, *5*) or the adenine ring (*6*–*8*), as done for εATP (*7*, *8*). In particular, ribose modification is widely used because of its smaller effect on the biochemical properties of ATPases and its simple synthesis. Fluorescent ATP analogues utilize the environment sensibility of the fluorophore in kinetic studies of ATPases. 3′(2′)-*O*-*N*-methylanthraniloyl-ATP (Mant-ATP) is a well-known ATP analogue. When Mant-ATP binds to myosin or kinesin, its fluorescence intensity is significantly enhanced (*1*, *9*). The rates of ATP binding and ADP release were determined using Mant-ATP and Mant-ADP with a stopped-flow apparatus (*10*). However, Mant-ATP exists as a mixture in which the mant group is linked to 2′-OH or 3′-position of ATP. The 2′- and 3′-isomers exist in a 2 : 3 mixture (*1*). The 3′-Mant-ADP isomer preferentially binds to the *Dictyostelium discoideum* myosin II motor domain *(30)*. The 2′- and 3′-hydroxyl-modified isomers show different fluorescence properties during ATP hydrolysis for kinesin ATPases (*10*, *11*). The ATP analogues, which have uniform structures, are useful for kinetic studies. 2′- or 3′-Deoxy-ATP was used to synthesize uniform fluorescently labelled ATP analogues (*10*).

Non-nucleotide ATP analogues that do not have the basic structure of ribose and an adenine ring, excluding the triphosphate moiety, were previously prepared. Kuwajima *et al.* (*12*) synthesized the non-nucleotide ATP analogue β-naphthyl triphosphate. The absorption spectrum of β-naphthyl triphosphate was different from that of β-naphthyl diphosphate in the range of 290–335 nm. It was shown that β-naphthyl triphosphate hydrolysis by myosin can be recorded continuously as a function of time by using a spectrophotometer. However, the characteristic properties of β-naphthyl triphosphate as a substrate for myosin were different from those of regular ATP (*12*).

On the other hand, another non-nucleotide ATP analogue, 2-[(4-azido-2-nitrophenyl)amino]ethyl triphosphate (NANTP), which was designed for the photoaffinity labelling of myosin ATPase, was shown to function as a normal substrate of myosin (*13*–*15*). Although the ATP analogue is not composed of ribose and an adenine ring moieties, the overall structure and the molecular size of NANTP are similar to those of regular ATP. Spin-labelled photoaffinity non-nucleotide ATP analogues were also synthesized to

monitor conformational changes in myosin during the contractile cycle of muscles (*16*). The non-nucleotide analogues were clearly shown to be good substrates for ATPase and sensitive probes to monitor conformational changes and ATP hydrolysis.

However, a fluorescently labelled non-nucleotide has not been synthesized yet. Therefore, in the present study, we designed and synthesized a novel fluorescent non-nucleotide ATP analogue, *N*-methylanthraniloylamideethyl triphosphate (MANTTP), for use in kinetic studies of ATPases. The characterization of MANTTP as a substrate for myosin ATPase and the kinetic parameters of ATP hydrolysis of skeletal muscle myosin utilizing fluorescence resonance energy transfer (FRET) between MANTTP and intrinsic tryptophan residues in the motor domain of myosin were studied. The non-nucleotide fluorescent ATP analogue was shown to be applicable for kinetic studies of ATPases.

Materials and Methods

Proteins

Skeletal muscle myosin was prepared from chicken breast muscle according to the method of Perry (*17*) and then digested with α-chymotrypsin to obtain subfragment-1 (S1), as described by Weeds and Taylor (*18*). α-Chymotrypsin was purchased from Sigma. F-actin was prepared from chicken fillet according to the alternative methods of Straub and Feuer (*19*), Mommaearts (*20*) and Spudich and Watt (*21*). Obtained myosin and F-actin were stored at 0°C until use.

Synthesis of ATP analogue

N-methylanthranilic acid (120 mM) was mixed with 1,1′-carbonyldiimidazole (Aldrich; 480 mM) in anhydrous *N,N*-dimethylformamide and incubated at room temperature for 30 min with shading and stirring. After activation with 1,1′-carbonyldiimidazole, the same volume of 120 mM *O*-phosphorylethanolamine aqueous solution was added by dropping and reacted at room temperature overnight. After removing the solvent, the reactant was dissolved in 5 mM triethylamine bicarbonate buffer (pH 7.0). *N*-methylanthraniloyl-amideethyl monophosphate (MANTMP) was purified using a diethylaminoethane (DEAE)-Sepharose column (GE Healthcare) and eluted with a linear gradient of triethylamine bicarbonate (0.005–0.3 M). After collecting fractions containing MANTMP, the solvent was removed using a rotary evaporator and lyophilization. MANTMP was stored at −30°C until use. The purity of the product was analysed by thin-layer chromatography (TLC) on silica gel plates (Silicagel 70 F_{254} Plate-*Wako*) by using 1-buthanol/acetic acid/H_2O (5 : 2 : 3, by volume) as the developing solvent, and the Rf value was 0.53. MANTMP was mixed with a 5 × molar excess of 1,1′-carbonyldiimidazole in anhydrous *N,N*-dimethylformamide and reacted at room temperature overnight with shading and stirring. The reaction was stopped by adding an 8 × volume of absolute methanol and incubated at room temperature for 30 min with shading and stirring. A 4 × molar excess of tributylamine pyrophosphate in anhydrous *N,N*-dimethylformamide was added by dropping and reacted at room temperature for over 24 h. After centrifugation at 1110*g* at 4°C for 10 min, an excess amount of absolute methanol was added to the supernatant. After removing the solvent, the reactant was dissolved in 5 mM triethylamine bicarbonate buffer (pH 7.0). MANTTP was purified using a DEAE-Sepharose column and eluted with a linear gradient of triethylamine bicarbonate (0.005–0.6 M). After collecting fractions containing MANTTP, the solvent was removed using a rotary evaporator and lyophilization. MANTTP was stored at −30°C until use.

The purity of the product was analysed by TLC on silica gel plates (Silicagel 70 F_{254} Plate-*Wako*) by using 1-buthanol/acetic acid/H_2O (5 : 2 : 3, by volume) as the developing solvent, and the Rf value was 0.29.

Kinetic studies

The binding rate of MANTTP to myosin was measured at 25°C with an SX-20 (Applied Photophysics) with an instrument dead time of 0.03 ms. The binding of MANTTP was monitored by fluorescent energy transfer with tryptophan as the donor. The excitation wavelength was 280 nm, and a 435 nm band-pass filter was used for the output.

Fluorescence measurement

All enzymatic assays were performed at 25°C. Steady-state fluorescence was measured at 25°C with an F-2500 fluorescence spectrophotometer (HITACHI, Tokyo). MANTTP fluorescence was measured at 430 nm with excitement at 330 nm. A complex of *N*-methylanthraniloylamideethyl diphosphate (MANTDP)-beryllium fluoride (BeFn) with myosin S1 was prepared by adding 5 mM NaF and 1 mM BeFn after complete hydrolysis of MANTTP by myosin S1.

Results

Preparation and characterization of MANTTP

We designed and synthesized a novel non-nucleotide fluorescent ATP analogue, MANTTP. Although the ATP analogue does not contain ribose and an adenine ring, its overall molecular shape overlaps with that of regular ATP (Fig. 1). The fluorophore moiety of MANTTP is at the identical position of the adenine ring of ATP, and the aminoethyl group fulfills the role of ribose.

First, we synthesized MANTMP under the conditions described in 'Materials and Methods' section. After the coupling reaction of *N*-methylanthranilic acid and phosphorylethanolamine, the reaction product was applied to anion exchange column chromatography to purify MANTMP. The elution pattern of the products on the DEAE-Sepharose column chromatogram is shown in Fig. 2A. TLC revealed that the second peak contained MANTMP. Subsequently, the isolated MANTMP was activated by 1,1′-carbonyldiimidazole and reacted with pyrophosphate, resulting in the synthesis of MANTTP. MANTTP was purified with a DEAE-Sepharose column. Isolated MANTTP was eluted in peak c as shown in Fig. 2B. The final product of MANTTP was identified by TLC, infrared (IR) spectra and fast atom bombardment-mass spectrometry (FAB-MS; data not shown). The synthetic yield of MANTTP was ∼30%.

The UV-visible absorbance spectrum of MANTTP is shown in Fig. 3A. The characteristic absorption maxima were observed at 218, 250 and 328 nm. The molar extinction coefficient at 328 nm, $e^{M}_{328} = 2727 \pm 173\,cm^{-1}\,M^{-1}$, was determined by quantitative analysis of inorganic phosphate after complete hydrolysis by using myosin according to the methods of Youngberg and Youngberg (*22*).

The fluorescence excitation and emission spectra of MANTTP are shown in Fig. 3B. The excitation and emission maxima of MANTTP in neutral buffer (pH 7.5) were 329 and 431 nm, respectively. These were shifted toward shorter wavelengths by ∼10 nm compared with Mant-ATP and toward longer wavelengths by ∼5 - 10 nm compared with *N*-methylanthranilic acid.

Interaction of MANTTP with myosin ATPase

Table I summarizes the divalent cation dependence of the hydrolysis of MANTTP by skeletal muscle myosin S1.

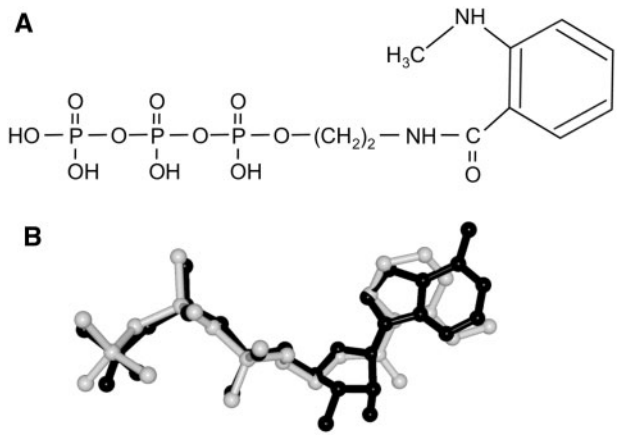

Fig. 1 Structure of MANTTP. (A) Structural formula of MANTTP. (B) Molecular model of MANTTP superimposed with AMPPNP in the crystal structure of 1KQM. MANTTP is shown in the grey ball and stick model, and AMPPNP is shown in black.

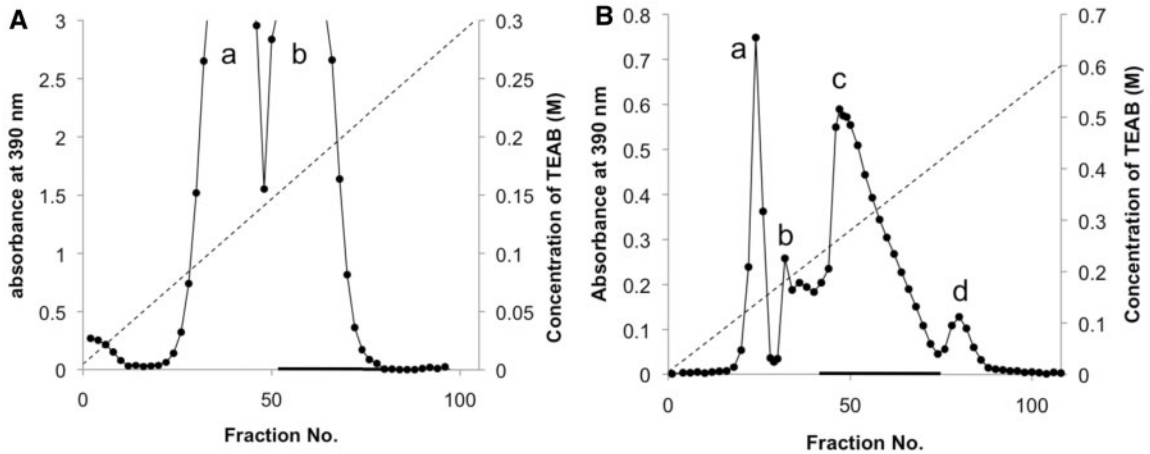

Fig. 2 Chromatographic purification of MANTMP (A) and MANTTP (B) on DEAE-Sepharose. (A) MANTMP linear gradient of 0.005–0.3 M TEAB. The solutions in tubes 51–78 were pooled and used in reactions as described in the text. (B) MANTTP linear gradient of 0.005–0.6 M TEAB. The solutions in tubes 44–74 were pooled.

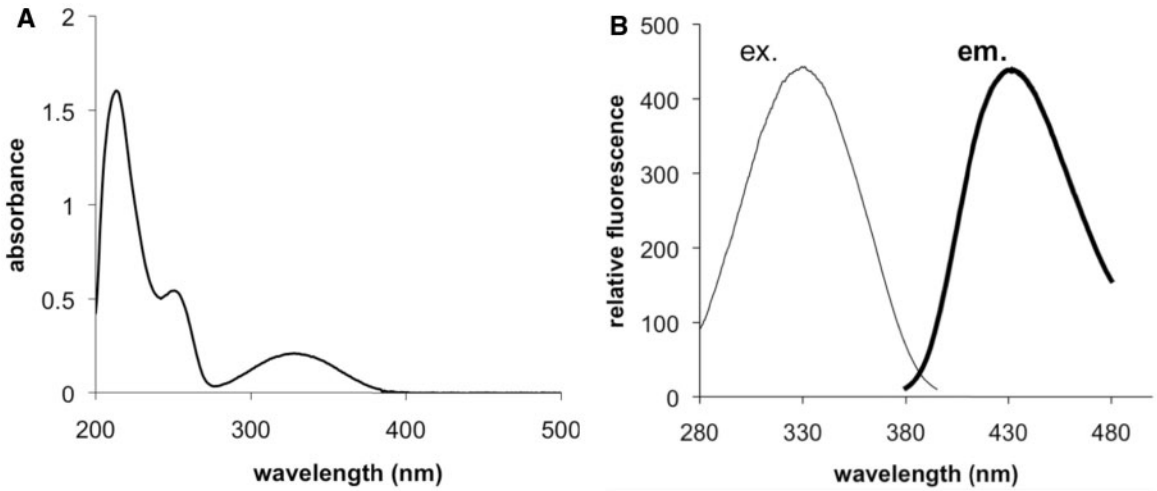

Fig. 3 Spectroscopic characteristics of MANTTP. (A) Absorption spectrum of MANTTP. The spectrum was measured in 30 mM Tris–HCl (pH 7.5), by using a U-3000 spectrophotometer (HITACHI) (B) Fluorescence excitation and emission spectra of MANTTP. Spectra were measured in 30 mM Tris–HCl (pH 7.5), at 25°C by using a RF-2500 fluorescent spectrophotometer (HITACHI). The excitation and emission wavelengths were 329 and 431 nm, respectively.

Table I. Divalent cation dependence of MANTTPase activity (Pi mol/S^{-1} mol/min).

	Mg^{2+} [a]	Ca^{2+} [b]	EDTA-K^{+} [c]	Actin-activated Mg^{2+} [d]
MANTTP	8.26	34.93	0.12	21.01
ATP	0.38	32.87	165.22	22.90

All reaction mixtures contained 30 mM Tris–HCl (pH 7.5) and 0.5 μM myosin S1.
[a]0.5 M KCl and 5 mM $MgCl_2$; [b]0.5 M KCl and 5 mM $CaCl_2$; [c]0.5 M KCl and 5 mM EDTA; [d]14 mM KCl, 2 mM $MgCl_2$ and 50 μM actin. The reaction was started by the addition of 2 mM MANTTP or ATP.

It is well known that myosin ATPase activity depends on the presence 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. On the other hand, in the presence of Mg^{2+} ions, MANTTP was hydrolysed ~21.7-fold faster than ATP. At the experimental condition of 2 mM MANTTP concentration, the hydrolysis rate showed almost V_{max}. In addition, the hydrolysis rate of Mg^{2+}-MANTTPase was accelerated ~2.5-fold by actin. The hydrolysis rate of Ca^{2+}-MANTTPase was slightly higher than that of ATPase. However, EDTA (K^{+})-MANTTPase was almost abolished. The characteristics of the unique divalent cation-dependent MANTTPase were similar to those of ATP analogues with the *syn* conformation with respect to the adenine-ribose bond (*23*, *24*).

Myosin-MANTDP-BeFn ternary complex

The formation of myosin-MANTDP-phosphate analogue ternary complexes, which mimic ATP hydrolysis transient states, was studied by measuring actomyosin dissociation. Actomyosin dissociation, as induced by MANTTP, was monitored by measuring light scattering. As shown in Fig. 4, the addition of MANTTP rapidly decreased the intensity of light scattering, which was accompanied by the dissociation of acto-S1. Subsequently, a rapid recovery of light scattering intensity was observed, which reflects the rapid hydrolysis of MANTTP to MANTDP. After complete hydrolysis of MANTTP, a phosphate analogue, BeFn, was added, and redissociation of actomyosin, reflecting the formation of the myosin-MANTDP-BeFn ternary complex, was observed.

Formation of the ternary complex was also examined by measuring quenching with acrylamide and fluorescence polarization of MANTDP in the ternary complex (Figs 5 and 6). As the change in the Stern-Volmer quenching constant (Ksv) reflects the accessibility of the fluorophores to solute quenchers and the change in fluorescence polarization reflects the changes in mobility of the fluorophore, MANTDP in the ternary complex can be discerned from free MANTDP. After complete hydrolysis of MANTTP by myosin, a phosphate analogue, BeFn or vanadate (Vi), was added to form ternary complexes. Subsequently, acrylamide

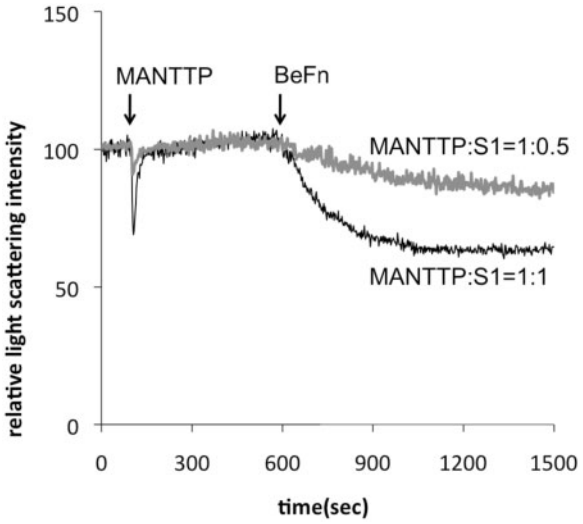

Fig. 4 Light-scattering observation of dissociation of acto-S1. MANTTP at 1 or 0.5 μM was added to a solution of 1 μM myosin S1, 2 μM actin, 30 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 100 mM KCl. Light scattering at 400 nm was observed using a HITACHI F-2500 fluorescence spectrophotometer at 25°C.

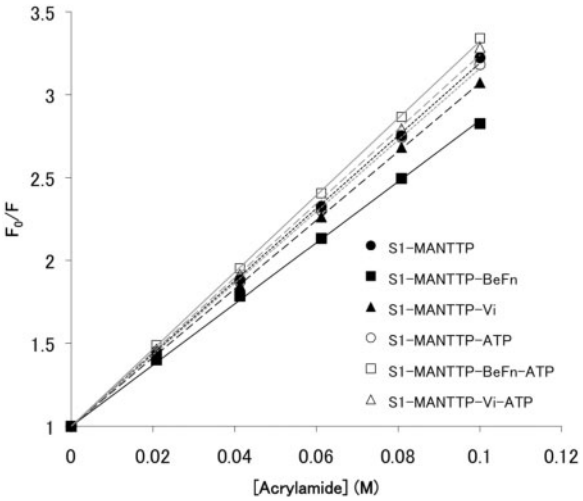

Fig. 5 Acrylamide quenching. F_0/F was plotted as a function of the acrylamide concentration. F_0 is the fluorescence observed in the absence of acrylamide. F is the observed fluorescence. All solutions contained 5 μM S1, 2.5 μM MANTTP, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl. The excitation and emission wavelengths were 330 and 430 nm, respectively.

was added, and after additional incubation, the Ksv was measured from the plots of relative fluorescent intensity versus acrylamide concentration plot.

In the presence of BeFn, Ksv of MANTDP for acrylamide decreased, and fluorescent polarization increased, reflecting the formation of the myosin-MANTDP-BeFn ternary complex. In addition, the presence of ATP competitively hindered the binding of MANTDP-BeFn to the active site of myosin. On the other hand, in the presence of Vi, both Ksv and polarization values were similar to those in the absence of phosphate analogues. Thus, it was demonstrated that MANTDP does not form a ternary complex with Vi.

Fluorescent studies on the interaction of MANTTP with myosin

As shown in Fig. 7A, the fluorescence intensity of MANTTP was not changed during hydrolysis by myosin S1. The fluorescence emission spectra of MANTTP in the presence of myosin and the myosin-MANTDP-BeFn ternary complex were almost identical to that of free MANTDP (Fig. 7B). This is inconsistent with the sensitive fluorescence changes of other fluorescent ATP analogues, *e.g.* 2′(3′)-*O*-NBD-ATP, 2′(3′)-*O*-Mant-ATP during ATP hydrolysis (*1*, *2*). The fluorescently insensitive property of MANTTP is advantageous for its application in FRET.

The fluorophore of the Mant group is known to be an acceptor of the fluorescence emission of tryptophan (*26*). FRET between MANTTP and the intrinsic tryptophan residues in the motor domain of myosin S1 was successfully observed during MANTTP hydrolysis. As shown in Fig. 8, the addition of myosin S1 to the solution of MANTTP immediately increased the fluorescence intensity of MANTTP emission at 430 nm after tryptophan excitation at 290 nm. Subsequently, the fluorescence intensity decreased according to MANTTP hydrolysis. The time of fluorescence change decreased, and the fluorescent intensity increased in a myosin S1 concentration-dependent

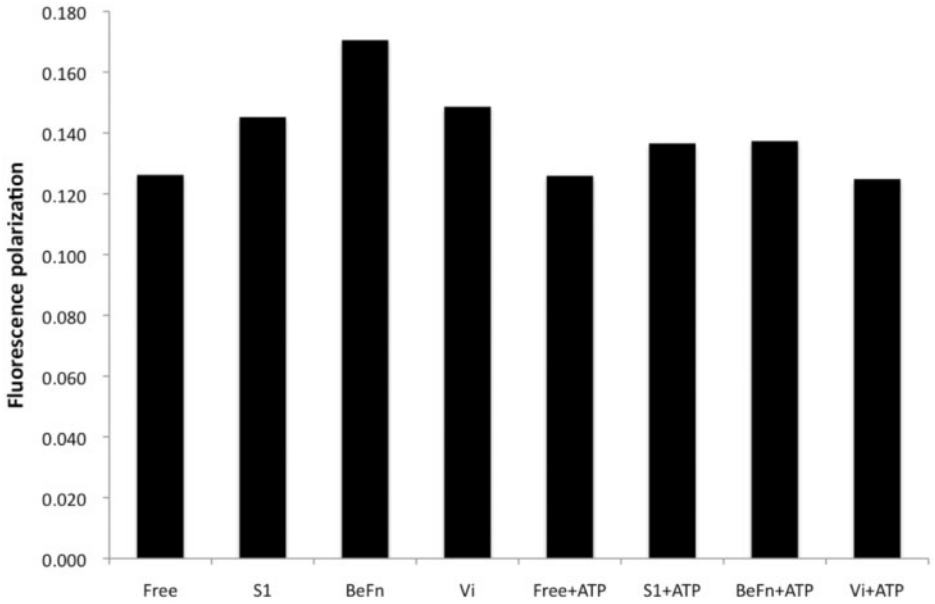

Fig. 6 Fluorescence polarization of MANTDP-S1-phosphate analogue complexes. All solutions contained 5 μM S1, 2.5 μM MANTTP, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl. The excitation and emission wavelengths were 330 and 430 nm, respectively.

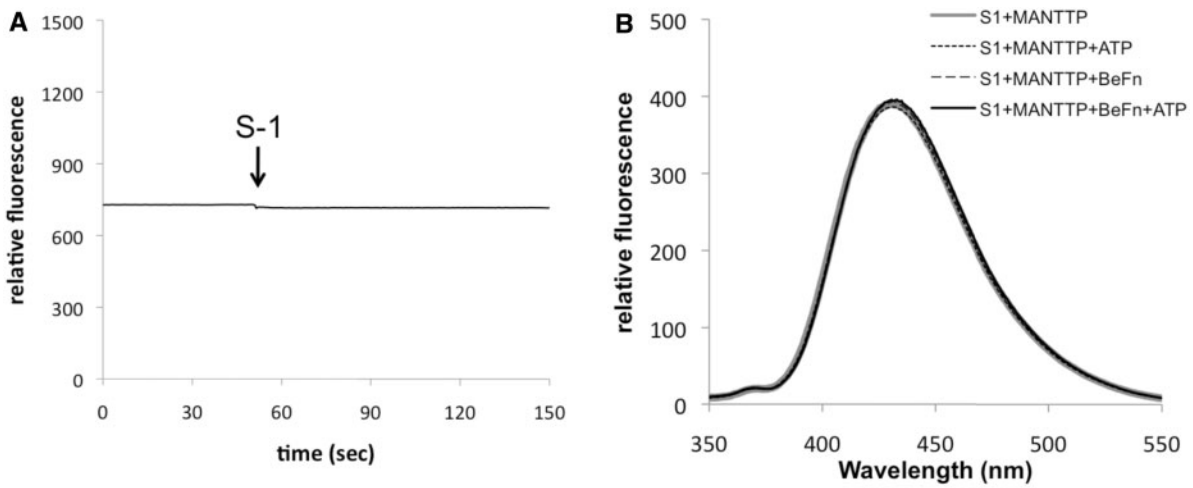

Fig. 7 Fluorescence emission of MANT interacting with myosin S1. (A) Time-course of the fluorescence change of MANTTP upon the addition of myosin S1. In a solution of 0.5 μM MANTTP containing 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl, 5 μM S1 was added, and the fluorescence of MANTTP was monitored (excitation at 330 nm and emission at 430 nm). (B) Emission spectra. All solutions contained 5 μM S1, 2.5 μM MANTTP, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl. After the reaction was completed, the fluorescence spectra were measured using a HITACHI F-2500 fluorescence spectrophotometer at 25°C. The excitation wavelength was 330 nm. The added BeFn concentration was 1 mM, and the ATP concentration was 5 μM. After adding ATP, the ATP-binding site should be completely occupied by ADP or the ADP-BeFn complex.

manner. These results suggested that MANTTPase could indeed be monitored by FRET between MANTTP and tryptophan in myosin S1.

The formation of the myosin-MANTDP-BeFn ternary complex was also monitored by the fluorescence change on FRET. As shown in Fig. 9A, fluorescence intensity on FRET was increased with the formation of the ternary complex. In the presence of excess ATP, FRET was significantly reduced. The fluorescence emission spectra of the ternary complex excited at 290 nm had a shoulder ~430 nm that was induced by FRET (Fig. 9B).

We also studied the kinetics of the first binding of MANTTP to myosin S1 under pseudo-first order conditions of [MANTTP]>[S1] by using a stopped-flow technique (Fig. 10).

MANTTP binding resulted in increased fluorescence intensity on FRET. The time course of the increased fluorescence was identical in successive pushes of the stopped-flow apparatus. The data were fitted to a single exponential curve. The typical plot is shown in Fig. 10A. The apparent second-order rate constant (0.15×10^{-6}), given as the initial slope of these plots (Fig. 10B), was 14-fold lower than that for ATP (2.1×10^{-6}).

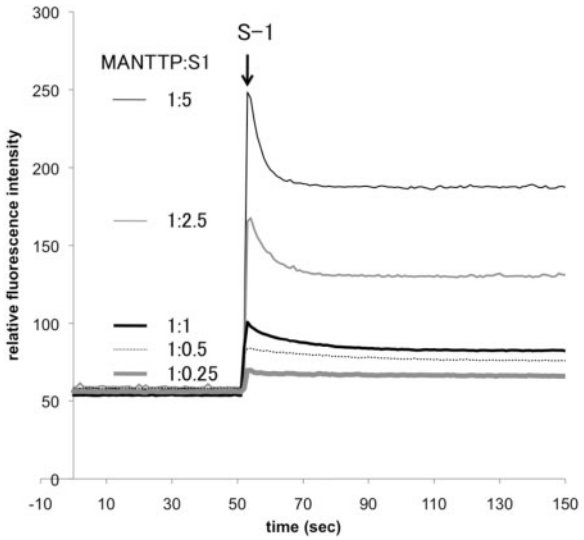

Fig. 8 Myosin S1 concentration dependency of MANTTP binding. Myosin S1 (0.25–5 μM) was added to a solution of 1 μM MANTTP, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl. The excitation wavelength was 290 nm, and the observed wavelength was 430 nm. These data were observed using a HITACHI F-2500 fluorescence spectrophotometer at 25°C.

Discussion

The aim of this study is to develop and characterize a novel fluorescent ATP analogue for application in kinetic and structural studies of ATPases.

We newly designed and synthesized a non-nucleotide fluorescent ATP analogue, MANTTP. The design of MANTTP was based on the structure of the non-nucleotide photoaffinity ATP analogue NANTP. Surprisingly, despite its relatively simple structure, NANTP has proven to be remarkably similar to ATP in its interaction with myosin and muscle fibers (*13–15*). Other related non-nucleotides based on NANTP have been also synthesized for the purpose of photoaffinity labeling and spin labeling (*16*, *27*). Some of these ATP analogues effectively mimic the

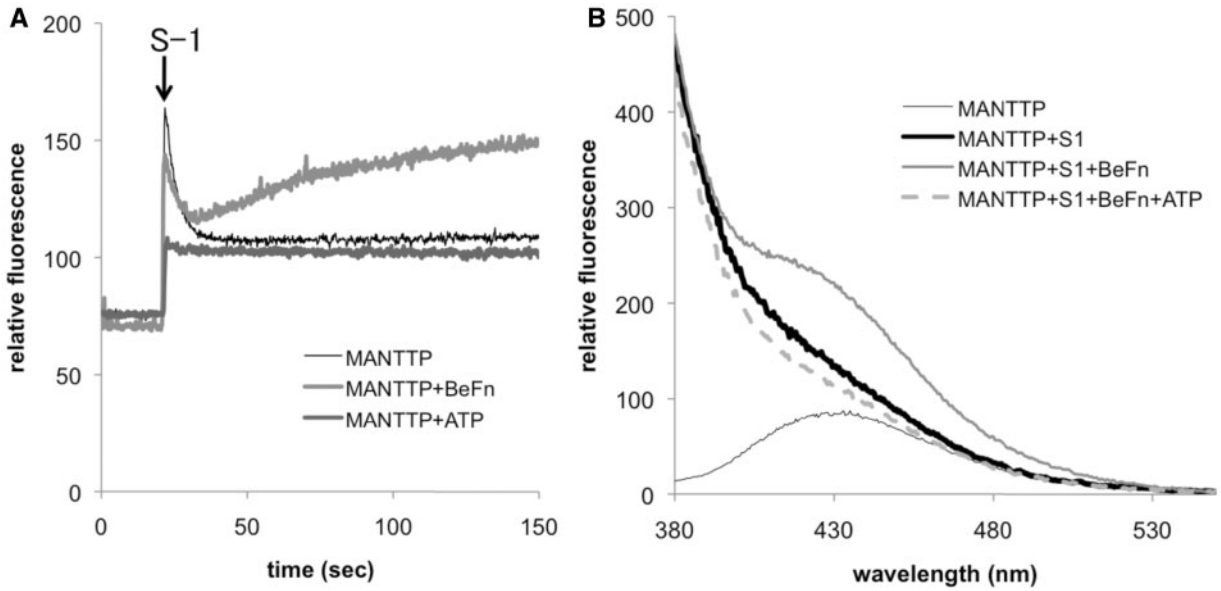

Fig. 9 Formation of S1-MANTDP-BeFn ternary complex. (A) Myosin S1 (5 μM) was added to the solution of 5 μM MANTTP, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl. In addition, the initial buffer contained ATP (gunmetal grey line) or 1 mM BeFn (grey line). The excitation wavelength was 290 nm, and the observed wavelength was 430 nm. These data were observed using a HITACHI F-2500 fluorescence spectrophotometer at 25°C. (B) All solution contained 5 μM MANTTP, 30 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 120 mM KCl. After the reaction was completed, the fluorescence spectra were measured using a HITACHI F-2500 fluorescence spectrophotometer at 25°C. The excitation wavelength was 290 nm. S1, BeFn and ATP were added at concentrations of 5 μM, 1 mM and 500 μM, respectively. After adding ATP, the ATP-binding site should be completely occupied by the ADP-BeFn complex.

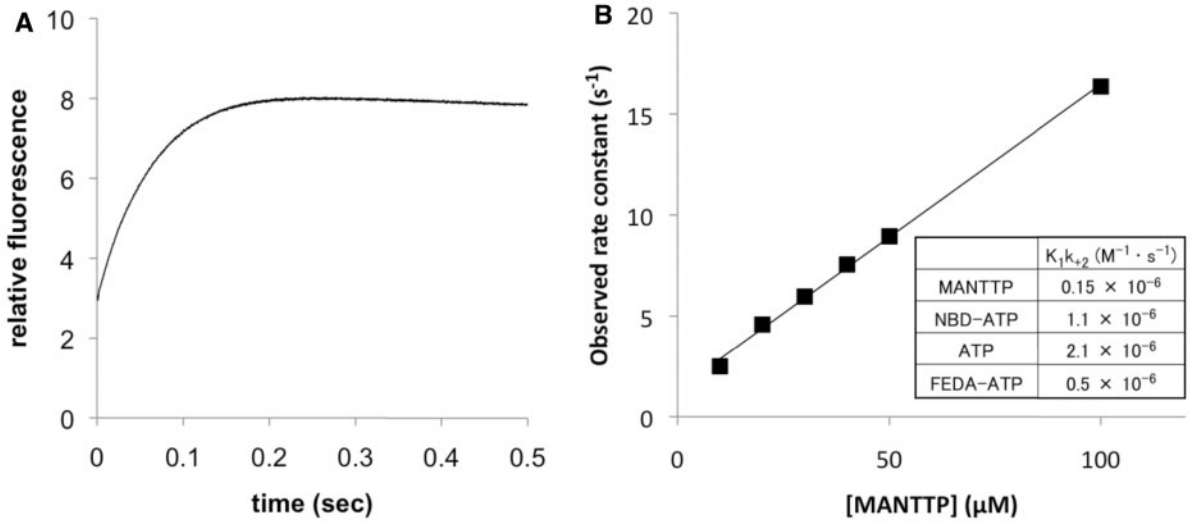

Fig. 10 Application of MANTTP for kinetic analysis using FRET with intrinsic tryptophan. (A) FRET measurement of 10 μM S1 and 100 μM MANTTP on the first binding. The buffer consisted of 40 mM NaCl, 20 mM Tris–HCl (pH 7.5) and 1 mM $MgCl_2$ and was incubated at 25°C. The observed rate constant was gained from the fitted single exponential curve from the result of 0.03 s to 0.15 s. $k = 16.36\,s^{-1}$. (B) MANTTP concentration dependence of the fitted rate constants. The apparent second-order rate constant, which was gained as the slope of these plots, was $0.151\ (\pm 0.003) \times 10^{-6}\,M^{-1}\,s^{-1}$.

chemical functionality of ATP for both ATPase activity and the ability to support muscle contraction (*15*). However, fluorescently labelled non-nucleotide ATP analogues have not been synthesized. Generally, the fluorescent probes were incorporated into the 2′ or 3′-*O* position of ribose in regular ATP via appropriate spacers for the synthesis of fluorescent ATP analogues, *e.g.* FEDA-ATP, 2′(3′)-*O*-[*N*-[2-[3-(5-fluoresceinyl)-thioureido]ethyl]-carbamoyl]adenosine 5′-triphosphate (*28*); Mant-ATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)-ATP (*5*); NBD-ATP, 2′(3′)-*O*-{6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic}-ATP (*2*); and REDA-ATP, 2′(3′)-*O*-[*N*-[2-[3-[5(6)-tetraethylrhodamine]-thioureido]ethyl]carbamoyl] adenosine 5′-triphosphate (*29*).

However, the existence of these ATP analogues as mixtures of 2′ and 3′ isomers hinders their application in kinetic studies. The non-nucleotide ATP analogues based on NANTP do not exist as mixtures of isomers. Moreover, due to the simple structure of NANTP, its analogs are readily synthesized on a large scale with inexpensive intermediate materials.

The synthesized MANTTP was a substrate for skeletal muscle myosin and was hydrolysed to MANTDP. The divalent cation dependence of MANTTPase activity is quite similar to that of ATP analogs with the *syn* conformation with respect to the adenine-ribose bond, *e.g.* 8-Br-ATP, 8-N_3-ATP (*23*, *24*). It is known that ATP is hydrolysed by skeletal muscle myosin via several transient state intermediates, M-ATP → M*-ATP → M**-ADP-Pi → M·ADP, as monitored by intrinsic tryptophan fluorescence changes (*25*). In the presence of Mg-ADP, myosin forms stable ternary complexes with Pi analogues, aluminum fluoride (AlF_4^-), BeFn and Vi. These ADP ternary complexes mimic the different transient states of each other. M-ADP-BeFn, M-ADP-Vi, and M-ADP-AlF_4^- resemble M*-ATP, M**-ADP-Pi, and M**-ADP-Pi → M·ADP, respectively (*2*). ATP analogues with the *syn* conformation form ternary complexes with BeFn but not with AlF_4^- or Vi (*24*). Therefore, ATP analogues with the *syn* conformation are hydrolysed by skeletal muscle myosin without formation of the M**-ADP-Pi state. Therefore, it is thought that MANTTP is also hydrolysed in a similar ATPase cycle to ATP analogues with the *syn* conformation. Indeed, as shown Figs 5 and 6, MANTDP formed a ternary complex with BeFn but not with Vi. MANTTP induced actomyosin dissociation but not actin gliding in an *in vitro* motility assay, which reflects the M*-ATP form but not the M**-ADP-Pi form during MANTTP hydrolysis. MANTTP may be applicable for the study of intermediates in the hydrolysis cycles of ATPases.

The ATP analogue Mant-ATP, possessing a Mant fluorophore with MANTTP in the 2′ or 3′ position of ribose, has significantly increased fluorescence intensity during hydrolysis by skeletal muscle myosin (*1*, *9*). In addition, NBD-ATP, which we have previously synthesized, exhibited decreased fluorescence intensity during hydrolysis (*2*). The fluorophore of the ATP analogues is near the ribose-binding region in the active site, where it is out of the ATP-binding site.

In contrast, as the fluorophore of MANTTP should be in the adenine-binding region of the active site, where Trp113 and Trp131 are located in skeletal muscle myosin, a significant fluorescence change was expected. However and unexpectedly, MANTTP showed novel fluorescent characteristics in that it does not exhibit changes in its fluorescence intensity and spectrum during interactions with skeletal muscle myosin. As shown in Figs 5 and 6, acrylamide quenching and fluorescence polarization experiments revealed that the fluorophore of MANTTP actually binds to the active site. MANTTP does not exhibit changes in its fluorescence intensity and the emission maximum during hydrolysis, which is advantageous for its

application in FRET. On the other hand, Mant-ATP that was previously well utilized for the kinetic studies changes its fluorescence significantly during its interaction with myosin. The significant change of fluorescence intensity and spectra makes the FRET analysis difficult. Therefore, Mant-ATP is not suitable for application in FRET studies. The other fluorescent ATP analogues showed similar fluorescent properties during interaction with myosin (*1*–*5*). For instance the emission maximum of 2′(3′)-O-[N-[2-[[[5-(dimethylamino)-naphthyl] sulphonyl]amino]ethyl]carbamoyl]adenosine 5′-triphosphate (DEDA-ATP) showed significant blue shift from 548 to 578 nm with change of fluorescence intensity when it binds to myosin (*1*). Instead of previous fluorescently labelled ATP analogues including Mant-ATP, MANTTP may be applicable to study the conformational changes of myosin by measuring the distance of fluorescent probes estimated from FRET. As the excitation spectrum of MANTTP overlaps with the emission spectrum of tryptophan, it is strongly expected that FRET occurs between MANTTP and intrinsic tryptophan residues near the ATPase site during hydrolysis. In fact, as shown in Fig. 8, significant FRET was observed when MANTTP bound to the ATPase site of skeletal muscle myosin. There are five tryptophan residues in skeletal muscle myosin at 113, 131, 440, 510 and 595. The tryptophan residues involved in FRET are thought to be 113 and 131, both of which are at approximately the same distance from the ATP-binding site.

In conclusion, the novel non-nucleotide ATP-analogue MANTTP was synthesized and characterized using myosin ATPase. MANTTP was hydrolysed by skeletal muscle myosin ATPase. MANTTP induced the dissociation of actomyosin and formed a myosin-MANTDP-BeFn ternary complex that mimics the transient states of the ATPase kinetic pathway. The fluorescence intensity of MANTTP was not influenced during myosin hydrolysis. Significant FRET between MANTTP and intrinsic tryptophan residues in the myosin motor domain was observed. MANTTP was shown to be a useful FRET probe for ATPase in kinetic and structural studies.

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Conflict of interest

None declared.

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