Interaction of Myosin Subfragment 1 with Two Non-Nucleotide ATP Analogues

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ABSTRACT: 2-[(2-Nitrophenyl)amino]ethyl triphosphate (NPhAETP) is the smallest ATP analogue that serves as a substrate for the actin-activated ATPase of myosin subfragment 1 (S1) and supports the development of active tension in skinned fibers. 2-(Phenylamino)ethyl triphosphate (PhAETP), in which the nitro group on the phenyl ring of NPhAETP is substituted by a H atom, is also a substrate of the actin-activated ATPase but does not support active tension [Wang, D., Pate, E., Cooke, R., and Yount, R. (1993) *J. Muscle Res. Cell Motil. 14*, 484−497]. We compared the S1-catalyzed hydrolysis of these analogues, their ability to support the formation of stable complexes with S1 and phosphate analogues, and their effect on S1 conformation. The analogues were hydrolyzed by S1 under various conditions both in the presence and in the absence of actin. In some cases, the effects of the two analogues are similar to each other and to those of ATP; they protect S1 from heat denaturation at 40 °C and inhibit the formation of the N-terminal 29 kDa fragment during the tryptic digestion of S1 and the modification of Lys-83 with trinitrobenzene sulfonate. However, in other cases, the effect of the two analogues is different; the effect of NPhAETP resembles that of ATP. NPhAETP and ATP decrease while PhAETP increases the rate of reaction of the SH_1 thiol (Cys-707) with coumarin maleimide. The diphosphate forms of the two analogues induce a much smaller change in the near-UV CD spectrum of S1 than ADP. NPhAEDP forms stable complexes with S1 in the presence of beryllium fluoride (BeF_x), aluminum fluoride (AlF_4^-), or vanadate (Vi) phosphate analogues, while the S1·PhAEDP complex is stable in the presence BeF_x but much less stable with AlF_4^- and Vi. These results indicate that the S1·PhAEDP·P_i state is poorly populated during the PhAETP hydrolysis. The models of the atomic structure of S1 complexed by the two analogues show that PhAETP, unlike NPhAETP or ATP, does not form a H bond with Tyr-134 in S1, which is the probable structural reason of the lack of tension development, with PhAETP as the substrate.

The molecular mechanism of muscle contraction and myosin-supported motility is based on the interaction of myosin with actin, coupled with ATP hydrolysis. The simplified four-step scheme for myosin-catalyzed hydrolysis of ATP (*1*) is depicted in Scheme 1

Scheme 1

$$M + ATP \leftrightarrow M^{*}\cdot ATP \leftrightarrow M^{**}\cdot ADP\cdot P_i \leftrightarrow M^{*}\cdot ADP \leftrightarrow M + ADP$$

where M, M*·ATP, M**·ADP·P_i, and M*·ADP represent conformational states of myosin−nucleotide complexes with distinct spectral properties.

Binding of ATP and the consecutive steps of its hydrolysis impose conformational changes on the active site of myosin. These structural distortions communicate with the actin binding site of the myosin head, called S1,[1] leading to well-defined changes in actin−myosin affinity at each step of the ATP hydrolysis. Among other techniques, substitution of ATP with other nucleotides (*2*, *3*) or non-nucleotide analogues (*4*, *5*) was used to elucidate structural changes taking place upon interaction of myosin, actin, and ATP. Rather simple non-nucleotide triphosphate ATP analogues (NTPs) have been shown recently to support actin−myosin interaction and tension generation in skinned muscle fibers (*4*). In these analogues, the purine or the pyrimidine base and the ribose ring were substituted by an aryl ring and an ethyl linker, respectively. The smallest of these analogues, which still supports tension generation and actin-activated myosin ATPase, is 2-[(2-nitrophenyl)amino]ethyl triphosphate (NPhAETP). A very similar analogue, 2-(phenylamino)ethyl triphosphate (PhAETP), in which the 2-NO_2 group on the phenyl ring of NPhAETP is substituted with a H atom, is a

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[1] Abbreviations: S1, myosin subfragment 1; NTP, non-nucleotide ATP analogue or ATP; NDP, non-nucleotide ADP analogue or ADP; PA, phosphate analogue; Vi, vanadate; BeF_x, beryllium fluoride complex; P_i, inorganic phosphate; AlF_4^-, aluminum fluoride complex; CPM, 7-(diethylamino)-3-(4′-dimaleimidylphenyl)-4-methylcoumarin; NPhAETP, 2-[(2-nitrophenyl)amino]ethyl triphosphate; PhAETP, 2-(phenylamino)ethyl triphosphate; TNBS, 2,4,6-trinitrobenzenesulfonate; PMSF, phenylmethanesulfonyl fluoride; CD, circular dichroism.

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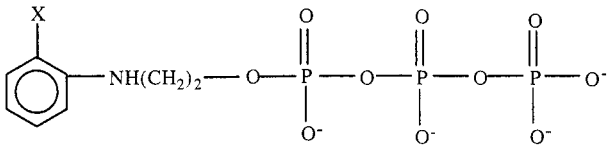

FIGURE 1: Structure of NPhAETP and PhAETP non-nucleotide ATP analogues. X shows the substitution at the 2 (ortho) position of the phenyl ring. X is NO_2 for NPhAETP and H for PhAETP.

substrate of the actin-activated myosin ATPase but does not support active tension (*4*). (The structure of these analogues is given in Figure 1.) Since a well-ordered occurrence of the consecutive steps of the ATP hydrolysis accompanied by defined conformational changes in the myosin head is required for active tension, comparing the steps of PhAETP hydrolysis and the accompanying structural changes to those of NPhAETP and ATP is of interest. By this comparison, we hoped to define the most important structural changes needed for the power stroke and tension development during muscle contraction.

In this work, the S1-catalyzed hydrolysis of PhAETP and NPhAETP was characterized in the presence and absence of actin under various ionic conditions. We tested the formation and stability of the complexes of S1 with PhAEDP and NPhAEDP together with the phosphate analogues BeF_x, AlF_4^-, and Vi. These analogues were used for studying the atomic structure of the various intermediate states of ATP hydrolysis (*6*, *7*). The general and localized conformational changes induced by the two non-nucleotide ATP analogues were also characterized. Our results suggest that because of the low population of the M**·ADP·P_i state during the myosin-catalyzed hydrolysis of PhAETP, this analogue does not support active tension. The structural cause of the deficient kinetic cycle can be the lack of H-bonding between this analogue and Tyr-134 of S1. A preliminary report of this work was presented at the 25th European Muscle Congress (*8*).

MATERIALS AND METHODS

Reagents. ATP, ADP, beryllium chloride, sodium vanadate, aluminum chloride, chymotrypsin, trypsin, phenylmethanesulfonyl fluoride (PMSF), and NaF were purchased from Sigma Chemical Co. (St. Louis, MO). 7-(Diethylamino)-3-(4′-dimaleimidylphenyl)-4-methylcoumarin (CPM) was purchased from Molecular Probes (Junction City, OR). TNBS and the reagents needed for the synthesis of NPhAETP and PhAETP were purchased from Aldrich (Milwaukee, WI). NPhAETP and PhAETP were prepared by the method of Wang et al. (*4*). All other chemicals were reagent grade. A stock solution of sodium vanadate (100 mM) was prepared according to the method of Goodno (*9*). Note that beryllium is very toxic and should be handled carefully. Fresh NaF stock solutions were prepared daily.

Proteins. Myosin and actin were prepared from back and leg muscles of rabbits (*10*, *11*). S1 was obtained by digestion of myosin filaments with chymotrypsin at a 300:1 (by mass) ratio (*12*). The digestion was stopped using 0.1 mM PMSF. Protein concentrations were obtained by absorbance, using an *A* (1%) at 280 nm of 7.5 for S1 and an *A* (1%) at 290 nm of 6.3 for actin. Molecular masses were assumed to be 115 and 42 kDa for S1 and actin, respectively.

NTPase Assays. Actin-, Mg^{2+}-, Ca^{2+}-, and K^+(EDTA)-activated NTPase activities, including NPhAETPase, PhAETPase, and ATPase activities (micromoles of phosphate per micromole of S1 per second), were calculated from the inorganic phosphate produced (*13*). The reactions were performed at 25 °C. For actin-activated NTPase, the assay contained 0.1 μM S1, 0−40 μM F-actin, 2 mM $MgCl_2$, 2 mM NTP, 14 mM KCl, and 20 mM HEPES buffer (pH 7.0). The same solution without actin was used for assays of the Mg^{2+}-modulated NTPase activities. For K^+(EDTA)-activated NTPase, the reaction mixture contained 0.2 μM S1, 2 mM ATP or NPhAETP or 5 mM PhAETP, 6.6 mM EDTA, 600 mM KCl, and 50 mM Tris-HCl buffer (pH 8.0). Ca^{2+}-activated NTPase was assayed under the same conditions as K^+(EDTA)-activated NTPase in the presence of 2 mM NTP using 5 mM $CaCl_2$ instead of EDTA.

Formation of Stable S1−NDP Complexes. S1 (20 μM) was incubated in 30 mM HEPES and 1 mM $MgCl_2$ at pH 7.0 and 25 °C with 0.5 mM NTP for 30 min. In the case of BeF_x- or AlF_4^--containing complexes, 5 mM NaF was also present. After that time, 0.2 mM Vi, 0.2 mM $BeCl_2$, or 0.8 mM $AlCl_3$ was added, and the incubation was continued at 25 °C for 30 min. Following the incubation, aliquots were taken at various time intervals to measure K^+(EDTA)-activated ATPase activity.

Dissociation of the Stable S1−NDP Complexes. The dissociation was studied by monitoring the dissociation of Mg^{2+} from the stable S1·MgNDP·Vi, S1·MgNDP·BeF_x, and S1·MgNDP·AlF_4^- complexes by EDTA chase. The released Mg^{2+} was chelated with EDTA, which reacts only with free Mg^{2+} but not with Mg^{2+} trapped in the complex. The EDTA-chelated Mg^{2+} cannot reenter to the complex, which decomposes in its absence. The S1·MgNDP·PA complexes were incubated at 25 °C in the presence of 4 mM EDTA, and the dissociation of the complex was followed by measuring the recovery of the K^+(EDTA)-activated ATPase activity.

Circular Dichroism Measurement. Near-UV CD spectra were recorded in a 10 mm path length cuvette at 20 °C in a Jobin et Yvon CD 6 spectropolarimeter. For each spectrum, at least four scans were averaged. The spectrum of 17 μM S1 in 1 mM $MgCl_2$ and 30 mM HEPES buffer (pH 7.0) was measured in the absence or in the presence of 0.2 mM NPhAEDP or ADP, or 1 mM PhAEDP. The CD spectrum recorded in the absence of S1 was subtracted from the spectrum of the S1-containing complex. The CD data for each wavelength were expressed as molar ellipticity, [Θ] (deg cm^2 $dmol^{-1}$):

$$[\Theta] = 100\Theta_\lambda/Cl$$

where Θ_λ is the measured ellipticity in degrees at a given wavelength, *l* is the optical path length in centimeters, and *C* is the molar concentration of S1.

Other Methods. Mild heat treatment of S1 was carried out according to the method of Setton et al. (*14*). The reactive lysine residue of S1 (Lys-83) was modified by TNBS (*15*). The limited trypsinolysis of S1 was carried out according to the method of Mornet et al. (*16*). SH_1 (Cys-707) was modified by CPM (*17*).

Table 1: Rates of S1-Catalyzed Hydrolysis of NTPs (s^{-1})

assay system[a]	ATPase	NPhAETPase	PhAETPase
Mg^{2+}	0.058 ± 0.007[b]	0.136 ± 0.019	0.018 ± 0.009
Ca^{2+}	0.53 ± 0.049	0.52 ± 0.068	0.30 ± 0.045
K^+(EDTA)	1.55 ± 0.18	0.34 ± 0.029	0.032 ± 0.006
actin, V_{max}	17.1 ± 1.82	4.0 ± 0.52	1.65 ± 0.021
actin, K_M (μM)	25.9 ± 2.48	3.55 ± 0.47	1.61 ± 0.35

[a] Assay conditions are described in Materials and Methods. [b] Mean ± SE ($n = 4$).

RESULTS

NTPase Activity. The hydrolysis of PhAETP, NPhAETP, and ATP by S1 was measured in the presence of Mg^{2+}, Ca^{2+}, and K^+(EDTA) in the absence of actin and with Mg^{2+} in the presence of actin (Table 1). Both ATP analogues are hydrolyzed by S1 as was shown previously (*4*). However, the activities observed in this study are quantitatively different from those obtained previously, which is probably due to different assay conditions. In each case in this work, the rate of hydrolysis was the highest with ATP and the lowest with PhAETP, except with Mg in the absence of actin where the rate of NPhAETP hydrolysis was the highest. However, large differences were observed between the activities measured under various conditions. In the presence of Ca^{2+}, the rates of hydrolysis of ATP and NPhAETP were essentially the same and the rate of the hydrolysis of PhAETP was only 40% slower than the rates with the former substrates. On the other hand, in K^+(EDTA)- and actin-activated NTPase activities, the rate of hydrolysis of PhAETP was only 2 and 10% of the ATP hydrolysis rate, respectively. Both the K_M and V_{max} values of the actin-activated activities significantly decreased in the order ATP > NPhAETP > PhAETP.

Formation and Dissociation of S1·MgNDP·Phosphate Analogue Complexes. S1 forms highly stable complexes with MgADP in the presence of the phosphate analogues Vi (*9*), BeF_x, and AlF_4^- (*18*, *19*). The formation of the complexes takes place at least in two steps; the first step is a rapid binding equilibrium, which is followed by a slow isomerization step (*19*, *20*) according to the following scheme:

Scheme 2

$$S1 + MgADP + PA \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} S1{\cdot}MgADP{\cdot}PA \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} S1^{\#}{\cdot}MgADP{\cdot}PA$$

$S1^{\#}$ indicates an altered conformation. We studied the formation of these stable complexes with ADP, NPhAEDP, and PhAEDP by following the loss of K^+(EDTA)-activated ATPase activity of S1 (*19*). The time course of the formation of the stable S1·NPhAEDP·BeF_x complex is shown in Figure 2. The formation of this and all the other complexes (not shown) follows a pseudo-first-order course described by a single exponential. The rate constants of the formation of the complexes, which denote the rate-limiting isomerization step, k_2, were calculated from the exponentials (Table 2). The production of the complexes containing NPhAEDP was slower than that of the respective ADP complexes, but the formation of each of the NPhAEDP complexes was essentially completed in 30 min. On the other hand, with PhAEDP, only the BeF_x complex formed in a significant quantity during the first 30 min; for the formation of the AlF_4^- complex, at least 120 min was necessary, while practically no Vi complex was formed.

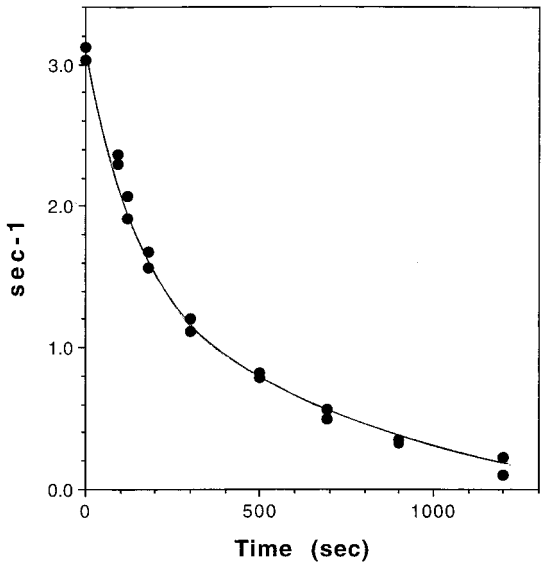

FIGURE 2: Formation of the S1·MgNPhAEDP·BeF_x complex. The formation was followed by monitoring the loss of the K^+(EDTA)-activated ATPase activity as described in Materials and Methods. The abscissa shows the ATPase activity expressed as P_i (M) per S1 (M^{-1}) per s^{-1}.

The S1·MgADP·PA complexes are stable, and their dissociation, which is followed by the recovery of the ATPase activity, takes place over the course of several days (*19*, *21*). The decomposition of the S1·MgADP·PA complexes is dramatically accelerated by actin (*19*, *21*−*23*), as that of the M**·MgADP·P_i complex, which is the rate-limiting step of the myosin ATPase (*1*). We also tested the effect of actin on the dissociation of the complexes of S1 with NPhAEDP and PhAEDP and phosphate analogues and found that the ATPase activity of S1 was rapidly recovered upon addition of actin (results not shown), like that observed with the ADP-containing complexes. The full recovery of the ATPase activity indicates that the loss of ATPase during the formation of NPhAEDP or PhAEDP and phosphate analogues containing complexes is not due to the denaturation of S1.

The spontaneous dissociation of the S1·MgNDP·PA complexes was studied by EDTA chase (see Materials and Methods). The time course of the dissociation is followed by monitoring the recovery of the K^+(EDTA)-activated ATPase activity (Figure 3). The time courses were fitted to a single exponential and characterized by a first-order kinetic constant, k_{-2}, which is the rate constant of dissociation (Table 2). The rate of dissociation of the S1·MgNPhAEDP·AlF_4^- complex is slow, similar to that of the S1·MgADP·AlF_4^- complex, and it takes 48 h to obtain a 84% recovery of the ATPase activity. On the other hand, the rate of decomposition of the S1·MgPhAEDP·AlF_4^- complex is fast and completed in 180 min. The dissociation of the BeF_x-containing complexes with both the two analogues and ADP is very slow (only the dissociation of the S1·MgNPhAEDP·BeF_x complex is shown). The progress of the recovery of S1 ATPase from these complexes has completely stopped

Table 2: Kinetic Parameters of the Formation and Dissociation of Stable S1·NDP·Phosphate Analogue Complexes[a]

	k_2 (s^{-1})	k_{-2} (s^{-1})	K_{assoc}
S1·MgADP·BeF_x	25.6×10^{-3} [b] $\pm 2.1 \times 10^{-3}$	$39.5 \times 10^{-6} \pm 4.7 \times 10^{-6}$	648 ± 57
S1·MgNPhAEDP·BeF_x	$3.9 \times 10^{-3} \pm 0.43 \times 10^{-3}$	$29.2 \times 10^{-6} \pm 2.1 \times 10^{-6}$	133 ± 21
S1·MgPhAEDP·BeF_x	$1.2 \times 10^{-3} \pm 0.09 \times 10^{-3}$	$11.7 \times 10^{-6} \pm 0.93 \times 10^{-6}$	103 ± 15
S1·MgADP·AlF_4^-	$4.0 \times 10^{-3} \pm 0.35 \times 10^{-3}$	$8.1 \times 10^{-6} \pm 0.75 \times 10^{-6}$	494 ± 53
S1·MgNPhAEDP·AlF_4^-	$0.93 \times 10^{-3} \pm 0.083 \times 10^{-3}$	$6.7 \times 10^{-6} \pm 0.53 \times 10^{-6}$	139 ± 17
S1·MgPhAEDP·AlF_4^-	$0.12 \times 10^{-3} \pm 0.017 \times 10^{-3}$	$396 \times 10^{-6} \pm 49 \times 10^{-6}$	0.32 ± 0.11
S1·MgADP·Vi	6.7×10^{-3} [d] $\pm 0.89 \times 10^{-3}$	16.4×10^{-6} [d] $\pm 2.13 \times 10^{-6}$	409 ± 39[d]
S1·MgNPhAEDP·Vi	$0.82 \times 10^{-3} \pm 0.74 \times 10^{-3}$	$485 \times 10^{-6} \pm 56.9 \times 10^{-6}$	1.7 ± 0.27
S1·MgPhAEDP·Vi	[c]	[c]	[c]

[a] Conditions for the stable complex formation and dissociation are described in Materials and Methods. [b] Mean ± SE ($n = 4$). [c] No formation of stable complex was observed. [d] Values are taken from Table 2 of ref *21*.

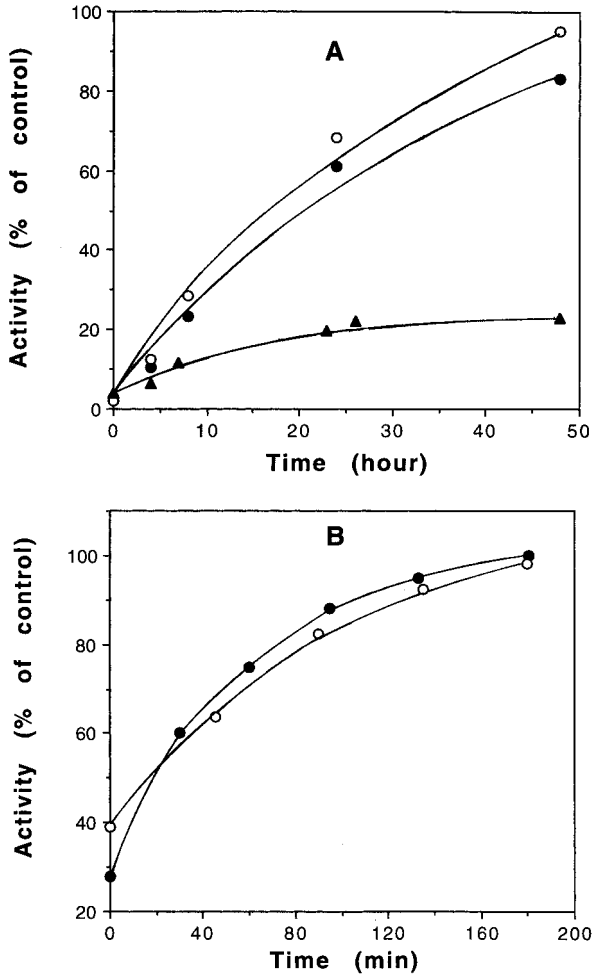

FIGURE 3: Dissociation of the S1·MgADP·AlF_4^-, S1·MgNPhAEDP·AlF_4^-, and S1·MgPhAEDP·AlF_4^- complexes. This was studied by monitoring the recovery of the K^+(EDTA)-activated ATPase activity as described in Materials and Methods. The abscissa shows the ATPase activity in the percentage of uncomplexed control S1: (A) S1·MgADP·AlF_4^- (●), S1·MgNPhAEDP·AlF_4^- (○), and S1·MgNPhAEDP·BeF_x (▲) and (B) S1·MgPhAEDP·AlF_4^- (○) and S1·MgNPhAEDP·Vi (●).

after 12 h and remained at a low constant level. This may be due to an additional isomerization step. A similar phenomenon was also observed with S1·MgADP·BeF_x (*18*). However, S1 also remained native in the BeF_x-containing complexes, since the ATPase activity was completely recovered following addition of actin even after a 60 h incubation at 25 °C in the presence of EDTA. The stability of the complexes was characterized by the association constant, *K*, which was calculated from the rate of formation

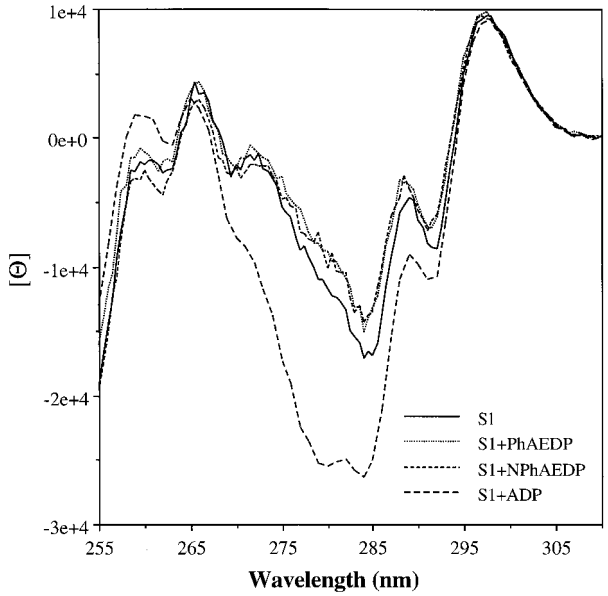

FIGURE 4: Near-UV CD spectra of S1 in the presence of NDPs. The near-UV CD spectra were recorded as described in Materials and Methods. The abscissa shows the [Θ], molar ellipticity (deg cm^2 $dmol^{-1}$).

and dissociation constants according to the equation $K = k_2/k_{-2}$ and presented in Table 2. The stability of the complexes decreases in the order ADP > NPhAEDP > PhAEDP. The S1·MgPhAEDP·AlF_4 and S1·MgPhAEDP·Vi complexes, which mimic the M**·ADP·P_i state, are especially very labile. The stability of the latter is so low that we could not even observe its formation by the stringent ATPase assay.

Effect of NTPs on the Near-UV CD Spectrum of S1. The near-UV CD spectrum of S1 in the 250−310 nm range is dominated by the contributions of the aromatic amino acids Phe, Tyr, and Trp (*24*). This spectrum has been found to be thoroughly influenced by ATP, ADP, and ADP−phosphate analogue complexes (*25*−*27*), which derive from conformational changes in the vicinity of aromatic residues located at or near the active site of myosin. On the other hand, addition of PhAEDP and NPhAEDP causes only minor changes, essentially a small increase in the 270−290 nm region of the S1 CD spectrum (Figure 4). This small change is not due to insufficient binding of the substrates since similar concentrations of PhAEDP and NPhAEDP caused significant change in the rate of modification of the SH_1 thiol of S1 (not shown).

Influence of NTPs on the Conformation of S1. This was studied by comparing the effect of the analogues with that

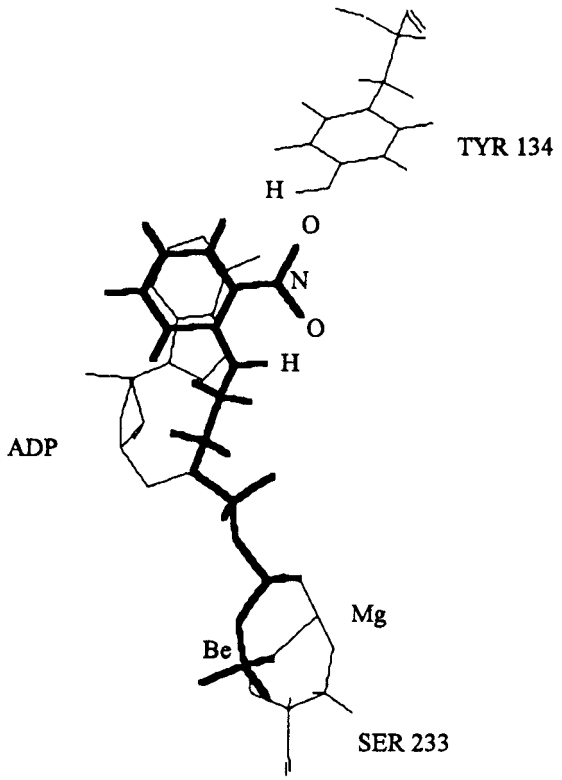

FIGURE 5: Molecular model of the interaction of the nitrophenyl moiety of NPhAETP with the nucleotide binding site of S1: thick line, the analogue structure; and thin line, the structure of S1Dc·MgADP·BeF_x according to Fisher et al. (6).

of ATP (i) on the rate of modification of Cys-707 and Lys-83 with CPM and TNBS, respectively; (ii) on the limited trypsinolysis; and (iii) on the heat denaturation of S1. In all these cases, with the exception of Cys-707 (SH_1) modification, the effects of analogues were similar to the effect of ATP (results not shown). In this latter case, it was found that the additions of NPhAETP and ATP decrease while the addition of PhAETP and all the NDPs increase the rate of the reaction relative to the rate obtained in the absence of ligand. The difference in the effect of ATP and NPhAETP and that of PhAETP can be best seen by comparing the ratio of the rate constants of the reaction in the presence of the respective di- and triphosphate forms: ADP/ATP, 1.64; NPhAEDP/NPhAETP, 1.67; and PhAEDP/PhAETP, 1.16.

Modeling the Structure of the S1−NTP Complexes. To reveal the structural basis of the different behaviors of the two ATP analogues in the actomyosin system, we modeled the active site of myosin containing ATP or ATP analogues. The basis of the model is the atomic structure of the truncated myosin S1 of Dictyostelium (S1Dc) complexed by MgADP·BeF_x (*6*). We used this structure since it closely mimics the structure of the ATP-bound state before the hydrolysis of ATP. The potential structure for the S1-bound NPhAETP (Figure 5) was found by using molecular mechanics energy minimization with MM+ force field (implemented on HyperChem molecular software, Hypercube, Inc., Waterloo, Ontario, Canada). In building the model, we assumed that the phosphate chains (Pα, Pβ, and Be or Pγ) in the crystal structure of S1Dc·MgADP·BeF_x and in S1·MgNPheAETP complexes are in identical positions. Next, we encouraged the proximity of the NO_2 group of the analogue and the OH group of Tyr-134 (rabbit skeletal S1 numbering) by applying a weak distance constraint between one oxygen in NO_2 and hydrogen in OH. The force constant of this distance constraint was reduced gradually to zero for the final energy minimization. Under these assumptions, we obtained a structure where the phenyl ring of the analogue maintains a position similar to that of the purine ring in the S1Dc·MgADP·BeF_x complex and where the oxygen in the nitro group of the analogue and the hydrogen in the hydroxyl group of Tyr-134 are in position to form a hydrogen bond. The O−H−O atoms in this apparent hydrogen bond form an angle of 164° and are separated by 3 Å. Since the second analogue, PhAETP, which does not support contraction, lacks the nitro group and, thus, cannot form H bonds with Tyr-134, it seems plausible that the H bond between the substrate and Tyr-134 has a crucial role in the proper functioning of the actomyosin system. The structure also has intramolecular H-bonding in the analogue between the second oxygen, which does not interact with Tyr-134, of the nitro group and the nearby hydrogen atom of NH in the aminoethyl grouping, which connects the phenyl ring with the triphosphate chain. It is also possible that this H bond has some significance in stabilizing NPhAETP in the nucleotide binding site of S1 since the methylation of NH, which eliminates the H-bonding, leads also to a loss of active tension (*28*) and formation of stable complexes with phosphate analogues (*5*).

DISCUSSION

NPhAETP is the simplest non-nucleotide ATP analogue, which is hydrolyzed by S1 and supports the tension development in skinned muscle fibers (*4*). Its derivative PhAETP, in which the ortho nitro group on the phenyl ring is substituted by a hydrogen, is a substrate of S1, but it does not support tension development (*4*). We compared the S1-catalyzed hydrolysis of ATP and these analogues, their ability to form stable complexes with S1 and phosphate analogues, and their ability to cause conformational changes in S1. By pinpointing the differences in the structural effects and mechanism of hydrolysis of PhAETP and NPhAETP (and ATP), we hoped to reveal the steps of the ATP hydrolysis and the structural features that are essential for energy transformation and force production.

Both similarities and differences were observed in the hydrolysis and structural effects of PhAETP, NPhAETP, and ATP. S1 catalyzed the hydrolysis of all three substrates in the absence of actin, and their Mg^{2+}-modulated hydrolysis was activated by actin. The rate of hydrolysis of PhAETP was in all cases slower than that of the other two substrates. Some of the structural changes in S1 caused by the three substrates were also similar. All the substrates protected S1 from denaturation by mild heat treatment. The tryptic cleavage at Lys-213 and the TNBS modification of Lys-83 of the S1 heavy chain were also equally well inhibited by the NTPs and ATP. Since both the proteolytic susceptibility and the rate of modification are structure-dependent properties, these results indicate that all three substrates induce similar conformational changes in the vicinity of the above lysine residues.

Besides the similarities, significant differences were also found in the effects of the NTPs. The near-UV CD spectrum of S1 was hardly affected by PhAEDP and NPhAEDP, while

it was strongly influenced by ADP in the tyrosine- and tryptophan-dominated 270−290 nm region. PP_i, like the analogues, did not affect the near-UV CD spectrum of S1 (*27*). The reason PhAEDP, NPhAEDP, and PP_i, which all lack the adenine ring, do not cause changes in the near-UV CD spectrum of S1 might be that the origin of the ADP-induced spectral change is the interaction of its adenine ring with the neighboring Tyr-134 and Trp-130 (*6*). Since NPhAEDP does not affect the near-UV CD spectrum of S1 but according to molecular modeling it interacts with Tyr-134, one may assume that the interaction of the adenine ring with the nearby Trp-130 is the reason for the change in the near-UV CD spectrum observed in the presence of ADP (*27*). It appears that the interaction of the adenine ring with Trp-130 is not essential for the tension development because NPhAETP supports development of tension (*4*) without altering the near-UV CD spectrum of S1.

NPhAEDP, like ADP, was found to support the formation of stable complexes with S1 in the presence of each of the three phosphate analogues. On the other hand, PhAEDP forms a stable complex with S1 only in the presence of BeF_x but not with AlF_4^- or vanadate. S1·MgADP·BeF_x mimics the M*·ATP state, while S1·MgADP·AlF_4^- and S1·MgADP·Vi model the M**·ADP·P_i state of the myosin-catalyzed ATP hydrolysis (*6*, *7*, *17*, *29*, *30*). Thus, the lack of formation of stable S1·MgPhAEDP·AlF_4^- and S1·MgPhAEDP·Vi complexes may indicate that the S1·MgPhAEDP·P_i complex is poorly populated during the hydrolysis of PhAETP. During the cross-bridge cycle, actin forms a weak complex with the M**·ADP·P_i intermediate. The existence of this complex in a significant concentration is a necessary precondition for the formation of the force-producing strongly bound actin−myosin complexes. Since in the hydrolysis of PhAETP the concentration of this intermediate is low, the formation of the force-generating complexes is precluded. Gopal et al. (*5*) came to a similar conclusion using a different pair of non-nucleotide ATP analogues, 2-[(2,4-dinitrophenyl)amino]ethyl triphosphate and its N-methylated derivative. Their nonmethylated derivative behaved like ATP, while the methylated derivative did not support the movement of actin filaments in the in vitro motility assay and the formation of stable S1 complexes with phosphate analogues, including BeF_x. Unlike the methylated derivative of Gopal et al. (*5*), PhAETP supported the formation of the S1·PhAEDP·BeF_x stable complex, which mimics the M*·ATP state. This would indicate that during the hydrolysis of PhAETP the S1·PhAETP intermediate is the predominant form. This assumption is supported by the very low rate of K^+(EDTA)-activated hydrolysis of PhAETP, where the actual hydrolysis step (step 2 in Scheme 1) is rate-limiting. The effects of analogues on SH_1 modification also indicate that the S1·PhAETP intermediate is the predominant form of the hydrolysis since in the presence of PhAETP the rate of SH_1 modification increased as in the presence of M*·ATP state-mimicking complexes (*17*). In this respect, PhAETP is similar to GTP because in the myosin-catalyzed hydrolysis of GTP the nonhydrolyzed triphosphate form is also the predominant intermediate (*3*).

We modeled the atomic structure of the active site of S1 containing each analogue and found that one of the oxygens of the NPhAETP's nitro group can form a H bond with Tyr-134 while PhAETP, which lacks the nitro group, cannot interact with Tyr-134. It seems that correct positioning of the nucleotide by H-bonding with Tyr-134 is necessary for the rotation of the γ-phosphate during the M*·ATP → M**·ADP·P_i transition (*6*, *31*), and therefore, the binding between the substrate and Tyr-134 is an essential structural feature of the proper functioning of the cross-bridge cycle. The importance of this bond has been predicted by Tonomura and his colleagues more than 30 years ago, when they concluded from their studies on ATP analogues that the binding of the substrate at a N or O atom of position 6 of the base with myosin probably through hydrogen bonding is necessary for contraction of myofibrils (*32*).

In summary, we conclude that the lack of tension development in the case of PhAETP is due to the poor population of the key M**·ADP·P_i intermediate state. The probable structural reason for the abnormal behavior of PhAETP is that this analogue, unlike NPhAETP or ATP, cannot form a hydrogen bond with Tyr-134 in the active site of S1. These results show how subtle differences in the substrate structure, such as substitution of a nitro group with a H atom, can profoundly affect the substrate−enzyme interaction and point to the usefulness of non-nucleotide ATP analogues in the elucidation of the molecular mechanism of contraction.

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REFERENCES

1. Bagshaw, C. R., and Trentham, D. R. (1974) *Biochem. J. 141*, 331−349.
2. Tonomura, M. (1973) *Muscle proteins, Muscle Contraction and Cation Transport*, pp 259−271, University Park Press, Baltimore, MD.
3. White, H. D., Belknap, B., and Jang, W. (1993) *J. Biol. Chem. 268*, 10039−10045.
4. Wang, D., Pate, E., Cooke, R., and Yount, R. (1993) *J. Muscle Res. Cell Motil. 14*, 484−497.
5. Gopal, D., Bobkov, A. A., Schwonek, J. P., Sanders, C. R., Ikebe, M., Levitsky, D. I., and Burke, M. (1995) *Biochemistry 34*, 12178−12184.
6. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) *Biochemistry 34*, 8960−8972.
7. Smith, C. A., and Rayment, I. (1996) *Biochemistry 35*, 5404−5417.
8. Moschcovich, L., Salomon, C., and Muhlrad, A. (1997) *J. Muscle Res. Cell Motil. 18*, 264−265.
9. Goodno, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2620−2624.
10. Tonomura, Y., Appel, P., and Morales, M. F. (1966) *Biochemistry 5*, 515−521.
11. Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem. 246*, 4866−4871.
12. Weeds, A. G., and Taylor, R. S. (1975) *Nature 257*, 54−56.
13. Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375−400.
14. Setton, A., Dan-Goor, M., and Muhlrad, A. (1988) *Biochemistry 27*, 792−796.
15. Muhlrad, A., and Takashi, R. (1981) *Biochemistry 20*, 6749−6754.
16. Mornet, D., Pantel, P., Audermard, E., and Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 98*, 923−932.
17. Phan, B. C., Peyser, Y. M., Reisler, E., and Muhlrad, A. (1997) *Eur. J. Biochem. 243*, 636−642.

18. Phan, B., and Reisler, E. (1992) *Biochemistry 31*, 4787−4793.
19. Werber, M. M., Peyser, Y. M., and Muhlrad, A. (1992) *Biochemistry 31*, 7190−7197.
20. Smith, S. J., and Eisenberg, E. (1990) *Eur. J. Biochem. 193*, 69−73.
21. Peyser, Y. M., Ben-Hur, M., Werber, M. M., and Muhlrad, A. (1996) *Biochemistry 35*, 4409−4416.
22. Goodno, C. C., and Taylor, E. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 21−25.
23. Phan, B. C., Faller, L. D., and Reisler, E. (1993) *Biochemistry 32*, 7712−7719.
24. Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113−175.
25. Murphy, A. J. (1974) *Arch. Biochem. Biophys. 163*, 290−296.
26. Chaussepied, P., Mornet, D., Barman, T. E., Travers, F., and Kassab, R. (1986) *Biochemistry 25*, 1141−1149.
27. Peyser, Y. M., Ajtai, K., Werber, M. M., Burghardt, T. P., and Muhlrad, A. (1997) *Biochemistry 36*, 5170−5178.
28. Wang, D., Pate, E., Cooke, R., Grammer, J. C., and Yount, R. (1995) *Biophys. J. 70*, A265.
29. Park, S., Ajtai, K., and Burghardt, T. P. (1996) *Biochim. Biophys. Acta 1296*, 1−4.
30. Ponomarev, M. A., Timofeev, V. P., and Levitsky, D. I. (1995) *FEBS Lett. 371*, 261−263.
31. Onishi, H., Morales, M. F., Kojima, S., Katoh, K., and Fujiwara, K. (1997) *Biochemistry 36*, 3767−3772.
32. Tonomura, Y., Imamura, K., Ikehara, M., Uno, H., and Harada, F. (1967) *J. Biochem. 61*, 460−472.

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