Adenosine 5′-(γ-Thiotriphosphate): An ATP Analog That Should Be Used with Caution in Muscle Contraction Studies†

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ABSTRACT: The slowly hydrolyzed ATP analog adenosine 5′-(γ-thiotriphosphate) (ATPγS) has been used in many studies of the muscle motor protein myosin in order to form a stable "weak binding" state analogous to the actin−S1−ATP complex. However, the results from studies using ATPγS do not always agree with the results of experiments using ATP. The binding of myosin subfragment-1-ATPγS to actin has now been studied in some detail to determine its relationship to the actin-S1−ATP state. The binding of myosin subfragment-1−ATPγS to actin−troponin−tropomyosin is similar in affinity to the binding of myosin subfragment-1−ATP. Like myosin subfragment-1−ATP, the binding is not Ca^{2+}-dependent, and most importantly, myosin subfragment-1−ATPγS does not stabilize the active configuration of actin−troponin−tropomyosin. Thus, myosin subfragment-1−ATPγS is an analog of myosin subfragment-1-ATP but must be used with caution for two reasons: (1) The binding of ATPγS to regulated actomyosin subfragment-1 is Ca^{2+}-sensitive, and errors can be made in the interpretation of results if proteins are not fully saturated with nucleotide and a mixture of weak and strong binding states is present. (2) At the high concentrations of myosin subfragment-1 used in some experiments, significant amounts of ADP may form. Since the binding constants of ADP and ATPγS to myosin subfragment-1 are similar, limited ADP production can lead to significant formation of the strong binding state myosin subfragment-1−ADP and stabilization of the active configuration of regulated actin.

Since the discovery that the complex of troponin and tropomyosin is responsible for the Ca^{2+}-mediated regulation of striated muscle contraction (for a review, see Ebashi *et al.*, 1969), numerous efforts have been made to determine which steps in the contractile cycle are inhibited in the absence of Ca^{2+}. The mechanism by which muscle contraction is regulated and the role of the weak-binding state have been difficult to assess, in part due to the rapid hydrolysis of ATP by actomyosin. This has led to the development of a number of nonhydrolyzable and slowly hydrolyzable ATP analogs such as adenosine 5′-(γ-thiotriphosphate) (ATPγS)[1] to help elucidate early steps in the ATPase cycle.

Studies in solution indicate that regulation of ATP hydrolysis is possible even without a change in the binding of S1−ATP to actin and suggest that troponin and tropomyosin regulate muscle contraction through an allosteric mechanism (for review, see Chalovich, 1992). The Ca^{2+}-dependent movement of tropomyosin (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973; Kress *et al.*, 1986) does not alter weak crossbridge binding to a great extent. Mechanical measurements in single muscle fibers indicate that the binding of myosin−ATP crossbridges to actin occurs in the absence of Ca^{2+} (Brenner *et al.*, 1982, 1991) even at near physiological conditions (Kraft *et al.*, 1995). However, similar single muscle fiber studies with ATPγS indicate that fiber stiffness, a measure of crossbridge attachment, increases as Ca^{2+} is increased, although no force is generated (Dantzig *et al.*, 1988). This result differed from that obtained with ATP and led to the conclusion that regulation of muscle contraction does involve steric blocking of weak-binding crossbridge states.

Given the different nucleotide-dependent results from muscle fiber studies, questions arise as to whether S1−ATPγS truly represents the weak-binding S1−ATP state or whether another factor such as a Ca^{2+} sensitivity in the binding of nucleotide to actomyosin could interfere with interpretation of results. Differences and similarities between ATP and ATPγS have already been documented. Unlike ATP, there is little actin activation of ATPγS hydrolysis (Goody & Mannherz, 1975), and in muscle fibers ATPγS hydrolysis is not Ca^{2+}-sensitive (Dantzig *et al.*, 1988). In addition, ATPγS has a lower rate of binding to and dissociating actomyosin than ATP (Goody & Hofmann, 1980). Furthermore, while the addition of ATP to S1 produces a mixture of states (S1−ATP and S1−ADP−P_i), the S1−ATPγS complex is thought to resemble S1−ATP. However, ATPγS is also reported to maintain the same open structure of weakly bound actomyosin formed with ATP as compared to the tight rigor complexes formed in the presence of ADP, AMPPNP, or PP_i (Duong & Reisler, 1989).

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[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; ATPase, adenosine triphosphatase; ATPγS, adenosine 5′-(γ-thiotriphosphate); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IANBD, (*N*-((2-(iodoacetoxy)ethyl)-*N*-methylamino)-7-nitrobenz-2-oxa-1,3-diazole; K_{ATP}, the apparent Michaelis−Menten constant for ATP expressed as an association constant; $K_{ATP\gamma S}$, the K_i for ATPγS expressed as an association constant; P_i, inorganic phosphate; PP_i, pyrophosphate; S1, myosin subfragment-1; TEA, triethylamine.

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Several biochemical criteria must be met if S1−ATPγS is to be considered a weak-binding state (Chalovich, 1992). First, the binding constant of S1−ATPγS to actin must be similar to that of S1−ATP. Second, the binding of S1−ATPγS to regulated actin must be insensitive to Ca^{2+}. In addition, the binding should not be cooperative and should not activate the thin filament. We now report that S1−ATPγS is a weak-binding state by these criteria. However, there are some disadvantages to using S1−ATPγS in place of S1−ATP. First, higher concentrations of ATPγS are required to saturate S1−actin−troponin−tropomyosin in the presence of Ca^{2+}. Therefore, care must be taken to ensure that enough ATPγS is used to mimimize the amount of nucleotide-free S1. This has been reported to be a serious problem in single fiber preparations (Kraft *et al.*, 1992). Second, ATPγS is hydrolyzed by S1, and the ADP formed competes with ATPγS for binding to S1, thus producing a population of strong-binding crossbridges.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was obtained from New England Nuclear (Wilmington, DE) and [^{3}H]succinimidyl proprionate from Amersham (Arlington Heights, IL). EDC was obtained from Pierce Chemicals (Rockford, IL), *N*-(1-pyrenyl)iodoacetamide and IANBD were from Molecular Probes, (Eugene, OR), and ATP and ATPγS were from Sigma (St. Louis, MO). ATPγS was purified by ion-exchange chromatography to remove ADP as described previously (Millar & Geeves, 1988), except that ATPγS was eluted with a gradient of 150−750 mM TEA−bicarbonate, pH 7.5. Purity was estimated by HPLC analysis to be greater than 96%. All other chemicals were reagent grade.

Protein Preparations. Skeletal actin was isolated from rabbit back and leg muscles as described by Spudich and Watt (1971) with minor modifications (Eisenberg & Kielley, 1972). Skeletal muscle myosin was isolated from rabbit back and leg muscles by the method of Kielley and Harrington (1960). Myosin subfragment-1 was prepared by digestion of myosin with chymotrypsin (Weeds & Taylor, 1975). The troponin−tropomyosin complex was prepared from an ether powder of rabbit muscle as described previously (Eisenberg & Kielley, 1974). When fluorescent probes were to be used, tropomyosin and troponin were isolated by chromatography on hydroxyapatite (Eisenberg & Kielley, 1974) or Affi-Gel blue (Reisler *et al.*, 1980), labeled, and reconstituted. Troponin was labeled with IANBD as described by Trybus and Taylor (1980); tropomyosin was labeled with *N*-(1-pyrenyl)iodoacetamide as described by Ishii and Lehrer (1990). Protein concentrations were determined by ultraviolet absorption at 280 nm, and the extinction coefficients used were 750 cm^2/g for S1, 1150 cm^2/g for F-actin, 290 cm^2/g for tropomyosin, 450 cm^2/g for troponin, and 380 cm^2/g for the troponin−tropomyosin complex. The concentration of cross-linked acto−S1was determined by the Lowry assay (Lowry *et al.*, 1951) with bovine serum albumin as the standard. For determination of molar concentrations, the following molecular weights were used: S1, 120 000; actin, 42 000; tropomysoin, 68 000; troponin, 80 000; and the troponin−tropomyosin complex, 150 000.

Preparation of Cross-Linked Acto−S1. EDC-cross-linked acto−S1 was prepared as described previously (Stein *et al.*, 1985; King & Greene, 1985) with minor modifications to obtain low levels of cross-linking. Actin at 60−70 μM was mixed with 0.75 mM EDC, and the cross-linking reaction was conducted with 0.6 μM S1 in 10 mM MES, pH 6.25, and 1 mM dithiothreitol at 5 °C for 9 min. The mole ratio of cross-linked S1 to actin obtained under these conditions was approximately 1:250; a trace amount of S1 labeled with [^{3}H]succinimidyl propionate (Rosenfeld & Taylor, 1984) was used to determine the mole ratio of cross-linked S1 to actin. Troponin−tropomyosin inhibited the ATPase activity of these cross-linked acto−S1 preparations by 85−95% in the presence of EGTA at low ionic strength (μ = 18 mM).

Binding Assays. The binding of S1−ATPγS to regulated actin in the presence and absence of Ca^{2+} was measured as previously described for the binding of S1−ATP (Chalovich & Eisenberg, 1982).

Stopped Flow Kinetic Studies. Measurements were made with an Applied Photophysics DX17.MV/2 sequential stopped flow spectrofluorometer. The binding of S1 or S1-nucleotides to regulated actin at low ionic strength in the absence of Ca^{2+} was monitored either as a decrease in IANBD-Tn fluorescence, monitored with a filter having 0% transmission at 510 nm and 80% transmission at 540 nm with excitation at 472 nm, or as an increase in pyrenyl-Tm fluorescence, monitored with a filter having 0% transmission at 450 nm and >80% transmission at 485 nm with excitation at 340 nm. Sequential mixing stopped flow was used to study the effect of nucleotide hydrolysis on the binding of S1-nucleotides to regulated actin. S1 was mixed with nucleotide, allowed to age for different periods of time, and then mixed with reconstituted thin filaments. Averages of at least three traces were analyzed with the software provided in the Applied Photophysics package.

ATPase Assays. Hydrolysis of ATP by S1 was measured by the rate of liberation of [^{32}P]P_i from [γ-^{32}P]ATP as described previously (Chock & Eisenberg, 1979). Assay conditions are given in the figure legends. When high actin concentrations were used in assays, the determination of [^{32}P]-P_i was improved by quenching reactions with KCl and excess cold ATP prior to acid precipitation (Hemric *et al.*, 1993). ATPase rates were calculated from a minimum of four time points taken during the initial 20% of the reaction, and rates were corrected for the ATPase activity of actin itself. The kinetic parameters K_m and k_{cat} describing the ATP dependence of rate data were determined from a direct fit to the Michaelis−Menten equation by the ENZFITTER (Elsevier-Biosoft) nonlinear regression program. For competitive inhibition studies with ATPγS, the kinetic parameter K_i was determined from a linear regression analysis of a plot of apparent K_m versus inhibitor concentration. The constants K_{ATP} and $K_{ATP\gamma S}$ are equal to $1/K_m$ and $1/K_i$, respectively, and have units of M^{-1} for ease of comparison with association constants. The very low MgATP concentrations used in this study necessitated the use of very low S1 concentrations (3−10 nM final). At these low S1 concentrations we observed somewhat less regulation of ATPase activity than in studies with final S1 concentrations of ≥0.1 μM.

ATPγS Hydrolysis. The hydrolysis of ATPγS catalyzed by S1 was measured by the rate of ADP formation as assessed by HPLC. A set of control reactions with ATP was also analyzed similarly. Assay conditions are given in the legend to Table 1. Reactions were quenched with perchloric acid, and nucleotides were extracted with 0.5 N trioctylamine in trichlorotrifluoroethane as described by

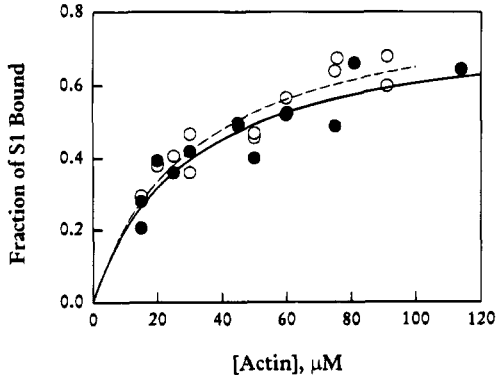


FIGURE 1: Binding of S1−ATPγS to regulated actin in the presence (●) and absence (○) of Ca^{2+}. Binding was determined by measuring the NH_4EDTA ATPase activity of free S1 after sedimentation of acto−S1 as described by Chalovich and Eisenberg (1982). The reaction conditions for binding were 1 mM ATPγS, 10 mM imidazole, pH 7.0, 3 mM $MgCl_2$, 0.5 mM EGTA or $CaCl_2$, and 1 mM DTT, 25 °C, μ = 17.5 mM. The S1 concentration was 0.35 μM. The ratio of troponin−tropomyosin to actin was 2:7. $K_{binding}$ is equal to 3.2×10^4 M^{-1} and 3.4×10^4 M^{-1} in the absence and presence of Ca^{2+}, respectively.

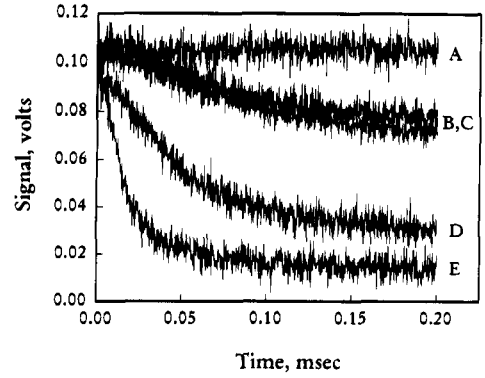


FIGURE 2: Binding of S1-nucleotides to actin−Tn*−tropomyosin as assessed by sequential mixing stopped flow. Fluorescence was measured as described in Materials and Methods. Final reaction conditions were 19 μM S1, 5 μM actin, 1 μM Tn*−tropomyosin, 10 mM imidazole, pH 7.0, 3 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, and 1 mM nucleotide at 25 °C. KCl was used to adjust the ionic strength to 22 mM. Cytochalasin D (0.01 mol/mol of actin) was added to facilitate mixing. (A) 1 mM ATPγS with 200 ms aging time with S1, (B) 1 mM ATPγS with 100 s aging time with S1, (C) 0.8 mM ATPγS plus 0.2 mM ADP, (D) 1 mM ATPγS with 200 s aging time with S1, (E) 1 mM ADP.

Sherman and Fyfe (1989). ADP and ATPγS were separated by ion-exchange HPLC on a Partisil SAX 10/25 column at a flow rate of 2 mL/min with a mobile phase of 0.5 M $NH_4H_2PO_4$, pH 4.8. The UV absorbance of the column effluent was monitored at 254 nm with an LDC/Milton Roy UV detector. Peak areas were integrated by a Spectra Physics SP4600 data jet integrator and were linearly related to the concentration of standards. Retention times were 2.8 min for ADP, 6.0 min for ATP, and 15 min for ATPγS. Hydrolysis rates were calculated from a minimum of four time points taken during the initial 20% of the reaction.

RESULTS

Both the affinity and the effect of Ca^{2+} on the binding of S1−ATPγS to actin−troponin−tropomyosin were measured at the low ionic strength used to characterize S1−ATP binding (Chalovich & Eisenberg, 1982). The results of this study are shown in Figure 1. The association constants obtained for S1−ATPγS binding to regulated actin are 3.2×10^4 and 3.4×10^4 M^{-1} in the absence and presence of Ca^{2+}, respectively. These association constants are similar to those reported by Chalovich and Eisenberg (1982) for S1−ATP (1.5×10^4 M^{-1}). These results suggest that S1−ATPγS does behave like the weak-binding state S1−ATP in terms of both the affinity and the Ca^{2+} sensitivity.

Perhaps the most distinguishing difference between weak and strong binding crossbridge states is that the latter are able to activate the actin−troponin−tropomyosin complex even in the absence of Ca^{2+}. Thus, with increasing concentrations of strong-binding crossbridge states in the absence of Ca^{2+}, there is a disproportionate increase in both the ATPase rate (Bremel *et al.*, 1972) and the binding of S1 to actin (Greene & Eisenberg, 1980). This change in the activity of the actin filament is also seen as a change in the fluorescence of probes on troponin (Trybus & Taylor, 1980; Greene, 1986) and tropomyosin (Ishii & Lehrer, 1990). We used the fluorescence change of IANBD-labeled troponin I (Tn*) or pyrene-labeled tropomyosin (Tm*) to monitor changes in the state of activation of the thin filament as S1−ATPγS binds to actin−troponin−tropomyosin. A lack of fluorescence change would support the idea that S1−ATPγS is a weak-binding crossbridge state.

When 38 μM S1 was mixed with 10 μM actin−Tm−Tn* in the presence of ATP in the stopped flow apparatus, there was no change in troponin I fluorescence. When the same experiment was done with 50 μM S1 and the ATP was replaced with ATPγS, there was a large change in fluorescence similar in magnitude to that seen in the presence of ADP or absence of nucleotide. This apparent activation by S1−ATPγS could be due to S1−ATPγS actually having the properties of a strong-binding crossbridge state or to the presence of sufficient ADP to activate the system.

ADP production can be a serious problem with ATPγS if high S1 concentrations are being used since ATPγS is hydrolyzed to ADP even in the absence of actin (Bagshaw *et al.*, 1972; also see Table 1 below). Since the affinity of ATPγS for S1 (10^7 M^{-1}) is weaker than that of ATP (10^{11} M^{-1}) and the affinity of ATPγS for S1 is only 10 times that of ADP (10^6 M^{-1}; Geeves, 1991), contamination with ADP is a much more serious problem in studies with ATPγS than in studies with ATP. Calculations of the simple competitve binding equilibria between ADP and ATPγS illustrate that when 20−40% of the ATPγS is converted to ADP, 2.5−6.4% of the S1 is bound to ADP. Since the binding of one S1 to seven actin monomers is sufficent to activate the thin filament, the hydrolysis of 20−40% of the ATPγS used in single mixing stopped flow studies is predicted to be sufficent to activate the thin filaments since the ratio of S1 to actin would be between 0.7 and 1.7 per 7 actin monomers.

To minimize ATPγS hydrolysis and confirm that hydrolysis of ATPγS to ADP could turn on the thin filaments, we compared the binding of S1−ATPγS and S1−ATP to actin−Tn*−tropomyosin by sequential mixing stopped flow. S1 (75 μM) was first mixed with 4 mM ATPγS or 4 mM ATP and allowed to age for 1 s. Less than 1% of the ATPγS or ATP was predicted to be hydrolyzed by S1 during the 1 s aging. This solution was then mixed with 10 μM actin−Tn*−tropomyosin, and the change in fluorescence was monitored. No change in fluorescence was obtained in the presence of ATPγS (Figure 2) or ATP (data not shown). A

Table 1: Steady-State Rates of Hydrolysis of ATP and ATPγS[a]

	k_{cat}, s^{-1}	
	ATP	ATPγS
S1	0.16	0.11
acto−S1	11.3	0.13
regulated acto−S1 + EGTA	0.21	0.18
regulated acto−S1 + Ca^{2+}	6.9	0.11

[a] Reaction conditions were 10 mM imidazole, pH 7.0, 3 mM $MgCl_2$, 1 mM ATP or ATPγS, 1 mM DTT, and 2 mM EGTA or 0.2 mM $CaCl_2$, 25 °C. Ionic strength was adjusted to 22 mM with KCl. Protein concentrations were as follows: S1, 0.1−2 μM; actin, 20 μM; and troponin−tropomysoin, 5.7 μM.

significant decrease in fluorescence was observed when sequential mixing stopped flow experiments were done in the presence of ADP (Figure 2) or absence of nucleotide (data not shown). Therefore, when the hydrolysis of ATPγS was minimized, there was minimal activation of the actin filament as would be expected for a weak-binding type crossbridge state. Similar results were obtained with actin−troponin−Tm* (data not shown).

To confirm that the formation of ADP was responsible for turning on the thin filament in the single mixing stopped flow studies, a series of sequential mixing studies were done with aging times varying from 1 to 200 s. From the rates of ATPγS hydrolysis given in Table 1, it was predicted that a 200 s aging time would lead to the hydrolysis of approximately 40% of the ATPγS. As shown in Figure 2, increasing the aging times for S1 and ATPγS led to increased turning on of the thin filaments. Similar results were obtained if ATPγS was spiked with a concentration of ADP predicted to be formed during the aging process (Figure 2). Therefore, the turning on of thin filaments in single mixing studies with effective aging times of greater than 180 s was most likely due to formation of ADP.

Since S1−ATPγS appeared to meet the criteria for a weak-binding state, we investigated whether Ca^{2+} itself affected the binding of ATPγS to regulated acto−S1. We estimated the affinity of ATPγS to acto−S1 by competitive inhibition of S1−ATPase activity. Biosca *et al.* (1986) have argued that this is the most accurate manner of determining the affinity of nucleotides to actomyosin. The method of Biosca *et al.* (1986) is valid for estimating a binding constant if a nucleotide analog is not hydrolyzed by S1 or if its hydrolysis is not Ca^{2+}-sensitive. If the rates of ATPγS hydrolysis were Ca^{2+}-sensitive, for example, the differences in K_i that were measured could be attributed to changes in the k_{cat} for ATPγS hydrolysis in addition to changes in the binding constant. The rates of ATPγS hydrolysis with S1, acto−S1, and regulated acto−S1 in the presence and absence of Ca^{2+} are given in Table 1. Rates of ATP hydrolysis estimated by the same procedure are included for comparison. There was minimal, if any, actin activation or Ca^{2+} sensitivity observed for ATPγS hydrolysis. Therefore, the method of Biosca *et al.* (1986) could be used for estimating a binding constant for ATPγS.

The ATP concentration dependence of actin-activated S1−ATPase activity was first measured with low concentrations of S1 and high concentrations of actin to promote the association of S1 to actin. From binding studies done under identical conditions to the ATPase assays, we estimated that >95% of the S1 was bound to actin under our conditions.

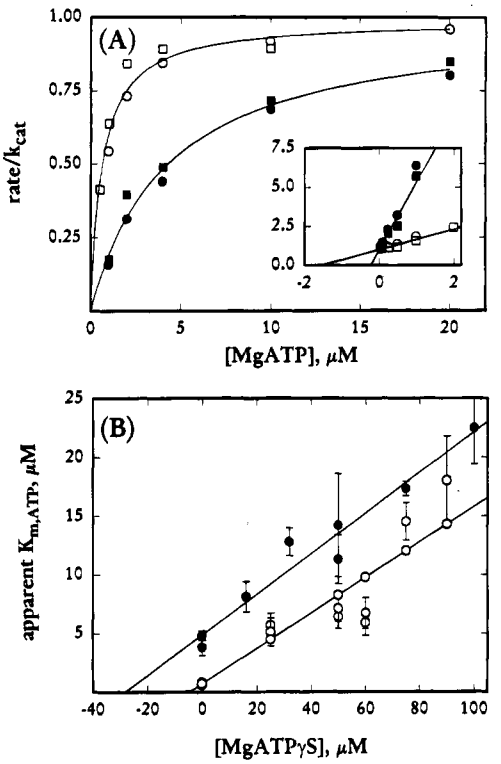


FIGURE 3: (A) Rate of ATP hydrolysis by regulated acto−S−1 in the absence (○, □) and presence (●, ■) of Ca^{2+} as a function of ATP concentration. Results are plotted relative to the k_{cat} for ease of presentation. The different symbols represent different protein preparations. The solid lines represent the best fit of each data set to a hyperbola. In the absence of Ca^{2+}, $K_{ATP} = 1.6 \times 10^6$ M^{-1} and $k_{cat} = 0.7 \pm 0.1$ s^{-1}. In the presence of Ca^{2+}, $K_{ATP} = 2.3 \times 10^5$ M^{-1} and $k_{cat} = 4.3 \pm 0.2$ s^{-1}. The reaction conditions were 10 mM imidazole, pH. 7.0, 2 mM free $MgCl_2$, 0.5 mM EGTA or $CaCl_2$, and 1 mM dithiothreitol, 25 °C. Ionic strength was adjusted to 14.5 mM with KCl. The S1 concentration was 3 nM, and actin concentration was 100 μM. The ratio of troponin−tropomyosin to actin was 2:7. MgATP concentrations ranged from 0.5 to 20 μM. The inset illustrates the linear double reciprocal plot generated from these data. (B) Binding of ATPγS to regulated acto−S1 in the absence (○) and presence (●) of Ca^{2+}. The binding of ATPγS was estimated functionally by the ability of ATPγS to inhibit the hydrolysis of the natural substrate ATP. The apparent $K_m \pm$ SE for ATP was plotted versus the concentration of ATPγS, and the data were fit by linear regression. In the presence of EGTA, $K_{ATP\gamma S} = 2.0 \times 10^5$ M^{-1}. In the presence of Ca^{2+}, $K_{ATP\gamma S} = 3.6 \times 10^4$ M^{-1}. Reaction conditions were as described above. MgATP concentrations ranged from 0.4 to 100 μM. MgATPγS concentrations ranged from 0 to 100 μM.

Figure 3A shows the ATPase rates as a function of the MgATP concentration at both high and low Ca^{2+} concentrations. Each data set is plotted relative to the maximum rate (k_{cat}) obtained with that particular protein preparation. This method of plotting facilitates the detection of differences in the K_m for ATP at high and low Ca^{2+} concentrations. The figure legends report the k_{cat} values obtained for each experiment; 7-fold differences in the k_{cat} values in paired experiments ± Ca^{2+} were typically obtained at low ionic strengths. The addition of ATPγS increased the apparent K_m for ATP without any significant change in k_{cat} (less than 2-fold), indicating that ATPγS was a competitive inhibitor of ATP hydrolysis. Figure 3B shows a plot of the apparent K_m values obtained for ATP as a function of the ATPγS concentration in the presence and absence of Ca^{2+}. The straight lines are least squares fits to the data. The inhibition constants for ATPγS are given by the absolute value of the

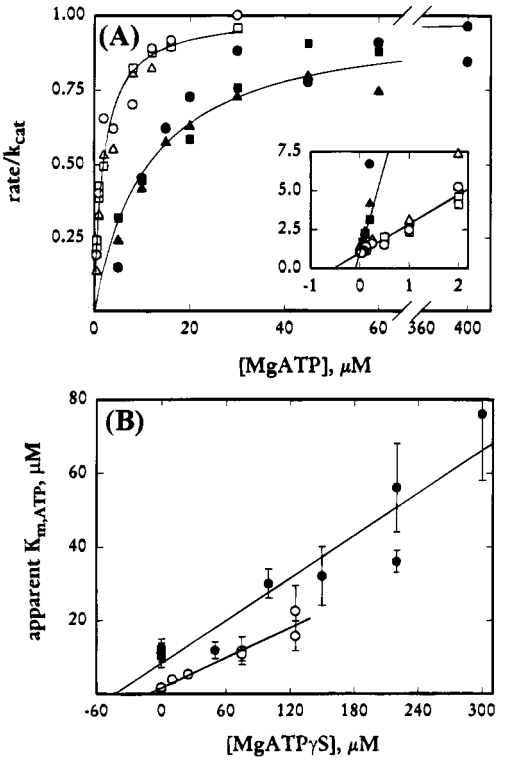


FIGURE 4: (A) Rate of ATP hydrolysis by regulated cross-linked acto−S1 in the absence (○, □, △) and presence (●, ■, ▲) of Ca^{2+} as a function of MgATP concentration. Results are shown relative to k_{cat}. The different symbols represent different preparations of both troponin−tropomyosin and cross-linked acto−S1. The solid lines represent the best fit of each data set to a hyperbola. In the absence of Ca^{2+}, $K_{ATP} = 5.3 \times 10^5$ M^{-1} and $k_{cat} = 0.7 \pm 0.1$ s^{-1}. In the presence of Ca^{2+}, $K_{ATP} = 9.1 \times 10^4$ M^{-1} and $k_{cat} = 4.3 \pm 0.5$ s^{-1}. The reaction conditions were as described in Figure 3. The final S1 concentration was 3 nM. The ratio of cross-linked S1 to actin ranged from 1:280 to 1:350. The ratio of troponin−tropomyosin to actin was 2:7. MgATP concentrations ranged from 0.5 to 100 μM. The inset illustrates the linear double reciprocal plot generated from this data. (B) Binding of ATPγS to regulated cross-linked acto−S1 in the absence (○) and presence (●) of Ca^{2+}. The binding of ATPγS was estimated as described in Figure 3. In the presence of EGTA, $K_{ATP\gamma S} = 8.3 \times 10^4$ M^{-1}. In the presence of Ca^{2+}, $K_{ATP\gamma S} = 2.0 \times 10^4$ M^{-1}. MgATP concentrations ranged from 0.5 to 300 μM. MgATPγS concentrations ranged from 0 to 300 μM.

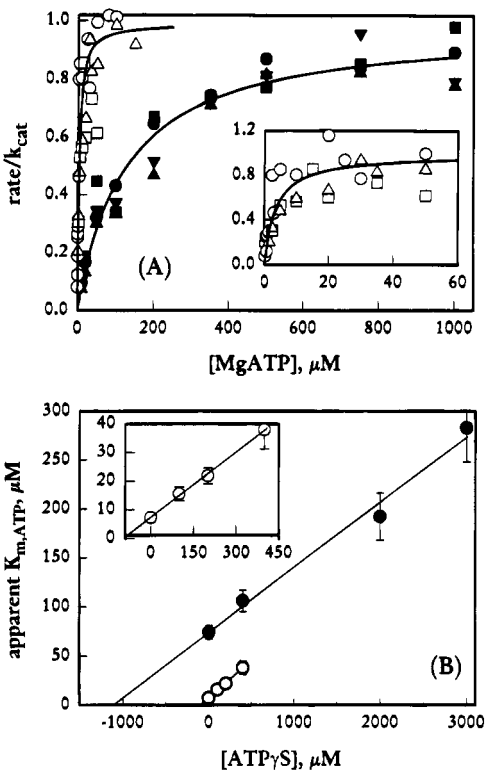


FIGURE 5: (A) Rate of ATP hydrolysis by regulated cross-linked acto−S1 at physiological ionic strength (μ = 170 mM) in the (A) absence (○, □, △) and presence (●, ■, ▲, ▼) of Ca^{2+} as a function of ATP concentration. Results are plotted relative to k_{cat}. The different symbols represent different protein preparations. The solid lines represent the best fit of each data set to a hyperbola. In the absence of Ca^{2+}, $K_{ATP} = 3.0 \times 10^5$ M^{-1}; k_{cat} values ranged from 0.26 to 0.78 s^{-1} for the three averaged sets of protein preparations. In the presence of Ca^{2+}, $K_{ATP} = 8.0 \times 10^3$ M^{-1}; k_{cat} values ranged from 11 to 23 s^{-1} for the four averaged sets of protein preparations. The reaction conditions were as described in Figure 3 except ionic strength was adjusted to 170 mM with KCl. The final S1 concentration was 5−10 nM. The ratio of cross-linked S1 to actin ranged from 1:280 to 1:350. The ratio of troponin−tropomyosin to actin was 2:7. MgATP concentrations ranged from 0.3 to 1000 μM. The inset is an expanded scale to highlight the data obtained in the absence of Ca^{2+}. (B) Binding of ATPγS to regulated cross-linked acto−S1 in the absence (○) and presence (●) of Ca^{2+} at physiological ionic strength. The binding of ATPγS was estimated as described in Figure 3. In the presence of EGTA, $K_{ATP\gamma S} = 1.0 \times 10^4$ M^{-1}. In the presence of Ca^{2+}, $K_{ATP\gamma S} = 9.0 \times 10^2$ M^{-1}. MgATP concentrations ranged from 1 μM to 3 mM. MgATPγS concentrations ranged from 0 to 3 mM. The inset is an expanded scale to highlight K_i data obtained in the absence of Ca^{2+}.

intercept on the abcissa. The results indicate that the removal of Ca^{2+} caused a 5-fold increase in the $K_{ATP\gamma S}$.

To ensure that the observed results were not biased by the presence of free S1, similar experiments were done with covalently cross-linked acto−S1. Such preparations have been shown to hydrolyze ATP at the maximal velocity so that only changes in the binding of nucleotides to the acto−S1 are observed. Figure 4A shows the ATPase activity as a function of ATP concentration for cross-linked acto−S1. In agreement with King and Greene (1985), the ATPase rate was sensitive to Ca^{2+} even though dissociation of S1 was impossible. This confirms that an allosteric component to regulation exists. Figure 4B shows the replot of apparent K_m values obtained from a series of curves as in Figure 4A but at various ATPγS concentrations. As with un-cross-linked proteins, increasing concentrations of ATPγS also increased the apparent K_m for ATP without any significant change in k_{cat} (less than 2-fold) when cross-linked proteins were used. Again, the association constant for ATPγS binding to cross-linked acto−S1 increased approximately 4-fold, from 2.2×10^4 M^{-1} to 8.3×10^4 M^{-1}, upon the removal of Ca^{2+}.

To determine whether the Ca^{2+} effect on ATPγS binding to regulated acto−S1 was an artifact of low ionic strength conditions, a series of ATPase measurements were done at physiological ionic strengths with covalently cross-linked acto−S1. Figure 5A shows the rate of ATP hydrolysis for regulated cross-linked acto−S1 at physiological ionic strength (μ = 170 mM) as a function of ATP concentration in the presence and absence of Ca^{2+}. The results obtained with several preparations of cross-linked acto−S1 and troponin−tropomyosin are shown. As before, the rate of ATP hydrolysis was Ca^{2+}-dependent even with cross-linked acto−S1. On average, the ATPase rate in the presence of Ca^{2+} was 36-fold greater than in the absence of Ca^{2+}. The addition of ATPγS weakened the apparent binding of ATP to S1. A replot of the apparent K_m for ATP against the

concentration of ATPγS is shown in Figure 5B. As was seen previously, there was a significant decrease in $K_{ATP\gamma S}$ in the presence of Ca^{2+}. The association constant for ATPγS binding to cross-linked acto−S1 increased approximately 11-fold, from 9.0×10^2 M^{-1} to 1.0×10^4 M^{-1}, upon the removal of Ca^{2+}. Therefore, the Ca^{2+} effect on ATPγS binding is even more pronounced at higher ionic strengths.

DISCUSSION

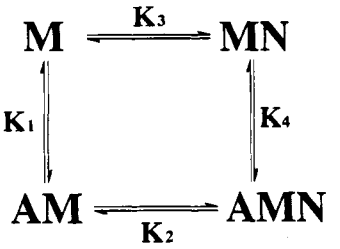
The binding of S1−ATPγS to actin−troponin−tropomyosin was observed to be similar in strength to that of S1−ATP and Ca^{2+}-insensitive at low ionic strengths. In addition, no signs of cooperative binding of S1−ATPγS to regulated actin were evident, as would be expected for strong-binding states. In particular, there was no change in the fluorescence of IANBD−troponin or pyrenyl−tropomyosin upon binding of S1−ATPγS to regulated thin filaments. The ability to activate the actin−troponin−tropomyosin complex is a primary determinant of whether a crossbridge is in a weakly or strongly bound state. Strong-binding crossbridge states such as S1, S1, S1−AMPPNP, and S1-PP_i can cause a cooperative transition in regulated actin (Bremel *et al.*, 1972; Greene & Eisenberg, 1980) while weakly bound crossbridges have little tendency to activate the actin filament (Chalovich *et al.*, 1983; Greene *et al.*, 1986).

The experiments of Duong and Reisler (1989) also lend support to the conclusion that S1−ATPγS behaves similarly to S1−ATP in solution studies. Tryptic digestion patterns, labeling patterns for reactive thiols, and enhancements of pyrenyl actin fluorescence are similar when ATP and ATPγS are bound to carbodiimide cross-linked acto−S1. Very different patterns are observed in the presence of ADP, AMPPNP, or PP_i. Kraft *et al.* (1992) have also reported that S1−ATPγS behaves as a weak-binding, S1−ATP like state in skinned muscle fibers. They found that when nucleotide is saturating, there is no increase in fiber stiffness and no detectable active force when ATPγS is added to relaxed fibers. In contrast, PP_i, a strong-binding state analog, does activate relaxed fibers. In addition, both stiffness−speed relationships at low Ca^{2+} concentration and equatorial diffraction patterns are similar with ATP or ATPγS as the bound nucleotide.

Since S1−ATPγS meets the general criteria for an S1−ATP like state in both solution and fiber studies, the observation of Ca^{2+}-dependent fiber stiffness by Dantzig *et al.* (1988) cannot be explained by the fact that ATPγS is not a weak-binding state. However, their conclusion that the binding of S1−ATPγS to the actin filament was blocked in the absence of Ca^{2+} could have been reached if ATPγS binding to myosin was Ca^{2+}-sensitive and myosin was not saturated with nucleotide under all conditions tested. A mixture of weak and strong binding states could lead to errors in interpretation of results.

Kraft *et al.* (1992) studied the effect of Ca^{2+} on ATPγS binding to crossbridges in skinned skeletal muscle fibers by monitoring changes in X-ray equatorial diffraction I_{11}/I_{10} X-ray intensities at varying ATPγS concentrations. They found that the concentration of ATPγS required to obtain a half-maximal intensity change in the I_{11}/I_{10} ratios is approximately 50- to 100-fold higher in the presence of Ca^{2+} than in the absence of Ca^{2+}. Our present results in solution also indicate that there is a significant decrease in the binding of ATPγS to acto−S1 in the presence of Ca^{2+}. However, there is a smaller Ca^{2+}-dependent change in nucleotide binding with S1 in solution (11-fold) than is observed with muscle fibers at physiological ionic strength.

Scheme 1



The Ca^{2+}-dependent binding of weak state nucleotides such as ATP or ATPγS to acto−S1 can be predicted as shown in Scheme 1 where M = myosin, A = actin, N = nucleotide, and K_x = a binding constant. By detailed balance $K_1K_2 = K_3K_4$. In the presence of ATP or ATPγS, neither K_3 nor K_4 is Ca^{2+}-dependent and the product of K_3 and K_4 is also Ca^{2+}-independent. Therefore, since Ca^{2+} increases the value of K_1, the value of K_2 must decrease proportionately. However, in the presence of a strong state nucleotide such as ADP, both K_1 and K_4 are Ca^{2+}-sensitive, which suggests that K_2 need not be Ca^{2+}-sensitive. Interestingly, when ADP was studied as a competitive inhibitor of ATP hydrolysis with cross-linked acto−S1 at physiological ionic strength, no difference in the K_i for ADP was observed plus or minus Ca^{2+} (data not shown).

That Ca^{2+} can regulate the affinity of nucleotides to acto−S1 is consistent with the findings of Rosenfeld and Taylor (1987). They demonstrated that the rate of release of nucleotides and products from regulated acto−S1 increases approximately 10- to 20-fold in the presence of Ca^{2+}. Under similar low ionic strength conditions, we observed a 5-fold increase in the binding constant of ATPγS for regulated actin in the absence of Ca^{2+}. Therefore, in the presence of Ca^{2+}, troponin−tropomyosin appears to accelerate the release of nucleotides by weakening the binding of nucleotides to acto−S1.

The Ca^{2+} effect on nucleotide binding must be accounted for in experimental protocols involving low nucleotide concentrations, especially for those nucleotides and nucleotide analogs which have a lower affinity for S1 than ATP. Otherwise, crossbridges will not be saturated with nucleotide and a mixture of crossbridge states may be present. It is also important to consider that the ADP produced from hydrolysis of ATPγS leads to the production of strong-binding S1 states. The severity of the problem was discussed in the context of Figure 2. Thus, while S1−ATPγS has properties which are similar to those of the S1−ATP state, ATPγS as a probe must be used with caution. It is important to ensure that the concentration of ATPγS is saturating in the presence of Ca^{2+} and that the ADP concentration is kept very low relative to the concentration of ATPγS for the duration of an experimental procedure.

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