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Inhibition of Myofibrillar and Actomyosin Subfragment 1 Adenosinetriphosphatase by Adenosine 5′-Diphosphate, Pyrophosphate, and Adenyl-5′-yl Imidodiphosphate

John Sleep* and Hilary Glyn
MRC Unit of Cell Biophysics, Kings College London, London WC2B 5RL, England
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ABSTRACT: Adenosine 5′-diphosphate (ADP), inorganic pyrophosphate (PP_i), and adenyl-5′-yl imidodiphosphate (AMPPNP) act as competitive inhibitors of the ATPase of myofibrils and actomyosin subfragment 1 (acto-S1). At I = 0.2 M, pH 7, and 15 °C, the inhibition constants for rabbit myofibrils are 0.17, 3, and 5 mM, respectively; the values for frog myofibrils at 0 °C are very similar, being 0.22, 1.5, and 2.5 mM. The inhibition constant of AMPPNP is about 2 orders of magnitude larger than the reported dissociation constant for fibers [Marston, S. B., Rodger, C. D., & Tregear, R. T. (1976) *J. Mol. Biol. 104*, 263–276]. A possible reason for this difference is that AMPPNP binding results in the dissociation of one head of each myosin molecule. The inhibition constants for rabbit acto-S1 cross-linked with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide measured under the same conditions were 0.12, 2.6, and 3.5 mM for ADP, PP_i, and AMPPNP, respectively. The inhibition of cross-linked and native acto-S1 was compared at low ionic strength and was found to be similar. The value for ADP is very similar to reported values of the dissociation constant whereas the inhibition constants for AMPPNP and PP_i are an order of magnitude weaker [Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255*, 543–548].

Knowledge of the steady-state ATPase properties of a muscle fiber is one of the requirements for understanding the relation between the unconstrained acto–myosin ATPase cycle in solution and the cycle coupled to work production in a muscle. As an extension of our work on the ATP dependence of the ATPase of rabbit psoas muscle fibers and myofibrils (Glyn & Sleep, 1985), we report in this paper an investigation of the inhibitory properties of products and substrate analogues. Each of the ligands chosen for study, adenosine 5′-diphosphate (ADP), inorganic pyrophosphate (PP_i), and adenyl-5′-yl imidodiphosphate (AMPPNP), tends to dissociate actin from subfragment 1 (S1); the binding constants of actin to S1 are reduced by factors of 20, 300, and 600, respectively (Greene & Eisenberg, 1980), values which are to be compared with the factor of 3000 resulting from ATP binding. AMPPNP binding has been reported to reduce the tension of a muscle fiber in rigor to about half, necessitating a stretch of about 2 nm per half-sarcomere to regain the original tension (Kuhn, 1973; Marston et al., 1976). ADP binding has a relatively small effect.

In simple models of the cross-bridge cycle [e.g., see Eisenberg & Hill (1985)], there are generally two attached states, the one before the power stroke (often being labeled 90°) and the one after the power stroke (45°). On this basis, the observed chemomechanical effect would be accounted for in terms of AMPPNP binding, tending to favor population of the 90° state rather than the rigor 45° state. However, from studies on the effect of AMPPNP on the structure of insect fibers, Reedy et al. (1983) suggested that AMPPNP binding may lead to one head of each myosin molecule dissociating. Data from several other sources, for example, digestion patterns of myofibrils (Chen & Reisler, 1984), orientation of spin-label (Thomas & Cooke, 1980), and fluorescence polarization (Yanagida, 1981), suggest that AMPPNP and PP_i binding results in the partial dissociation of myosin heads. Thus, the observed chemomechanical effect could also be accounted for in terms of AMPPNP reducing the binding constant of myosin heads for actin to the extent where strained heads become dissociated whereas unstrained heads remain bound. Dissociation of the strained head necessitates stretching of the muscle to regain the original tension. Comparison of the inhibition constants of myofibrillar ATPase with the dissociation constant of myofibrils and fibers offers a useful approach to clarifying this issue. If AMPPNP binding tends to dissociate one head from actin, then the AMPPNP dissociation constant of this head will be small and thus easily measurable. The behavior of this easily dissociated head will tend to dominate the binding behavior of fibers. In the case of inhibition studies, the opposite effect is operative. Heads which bind to actin have a much higher ATPase, and thus the ATPase measurements which give the inhibition constant report primarily on these active heads which display a high inhibition constant. Our observation that the inhibition constants for AMPPNP and PP_i were not equal to the reported values of the dissociation constants is thus consistent, at least in part, with this model in which AMPPNP and PP_i binding results in the dissociation of one head. The model accounts for the chemomechanical response of AMPPNP binding in terms of tension reduction because on each myosin the head that is under the greater strain becomes dissociated and not because of a shift in the population *of heads* from 45° to 90° states.

* Address correspondence to this author.

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In the case of actomyosin subfragment 1 (acto-S1), all heads operate in an identical environment, and thus, it is of interest to see whether the inhibition constant is equal to the dissociation constant. The inhibition constant of ADP was similar to the reported binding constant whereas the inhibition constants of PP_i and AMPPNP were an order of magnitude larger, an observation which cannot be accounted for by tinkering with kinetic schemes. However, Biosca et al. (1985) have reinvestigated the binding of AMPPNP and PP_i, and this more detailed study has shown that the dissociation constants are indeed much higher than previously reported, about 2 mM, in satisfactory agreement with the inhibition constants.

Materials and Methods

Materials. Myofibrils from rabbit psoas muscle and from the leg muscles of frog were prepared by tying 3-mm-diameter bundles to Perspex sticks and leaving overnight in ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)–Ringer's solution, followed by homogenization in a rigor solution as described by Trinick and Knight (1982). Myofibrils were cross-linked with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) to prevent sarcomere shortening (Glyn & Sleep, 1985). Myosin subfragment 1 was prepared from rabbit back and leg muscles by the method of Weeds and Taylor (1975). Actin was prepared by the method of Spudich and Watt (1971). Subfragment 1 was cross-linked to actin with EDC by using Rosenfeld and Taylor's (1984) modification of the method of Mornet et al. (1981).

Methods. The methods for measuring the ATPase of myofibrils by using a linked assay system have already been described (Glyn & Sleep, 1985). This method is useful for measuring the inhibition by PP_i, but any method involving ATP regeneration from ADP is fundamentally incompatible with measurement of ADP inhibition. In practice, the method is not satisfactory for measuring AMPPNP inhibition because the ratio of AMPPNP to ATP needed to get significant inhibition results in the ADP introduced as a contaminant of AMPPNP being greater than the desired ATP concentration. The resultant ATP concentration will not be known with high accuracy.

Inhibition by the three ligands was measured by following the first 25% of hydrolysis of [^{32}P]ATP in the absence of a regenerating system. It quickly became apparent that PP_i and AMPPNP were poor inhibitors and that to get significant inhibition low concentrations of ATP were needed. The ATP concentration chosen was about half the K_m as the extra inhibition at lower concentrations is very slight and other problems arise at very low concentrations. Each runs was started by the addition of 100 μL of a solution containing [^{32}P]ATP and the inhibiting ligand to 600 μL of myofibrils in a stirred vial held in a thermostated aluminum block. At 5 times, a 100-μL sample was quenched in 0.5 mL of 0.5 M perchloric acid, 4 mM phosphate, and 100 μM unlabeled carrier ATP in 1.5-mL Eppendorf centrifuge tubes; a further sample was taken for the total counts. The quenched samples were vortexed, 0.4 mL of 50 mg/mL activated charcoal was added, and they were vortexed again and centrifuged in an Eppendorf microfuge. A 0.75-mL sample of the supernatant was added to 2 mL of water and ^{32}P counted by Cerenkov radiation. The rates were obtained from a regression line to all points for which <25% of the ATP had been hydrolyzed. The myofibril concentration was determined by solution in hot 1% sodium dodecyl sulfate (SDS) and by taking the absorption coefficient as $E^{1\%}_{280-320} = 7.0$ (Trinick & Knight, 1982). The rates were converted from micromoles of ADP per milligram of myofibrils per minute to moles of ADP per mole of myosin heads per second by using the value 0.82 μmol of myosin/g of myofibrils (Yates & Greaser, 1983). Inhibition was found to be competitive and was analyzed in terms of $v = V_m[MgATP]/(K_m' + [MgATP])$ in cases where the MgATP concentration was varied [where $K_m' = K_m(1 + [I]/K_i)$] and in terms of $v = v'/(K_i' + [I])$ in cases where the ligand concentration was altered [where $K_i' = K_i(1 + [MgATP]/K_m)$ and $v' = K_iV_m[MgATP]/K_m$]. Experiments on rabbit preparations were done at 15 °C, and the basic activating solution was 3 mM $MgCl_2$, 30 mM CaEGTA, and 50 mM imidazole at pH 7 to which the stated concentrations of ATP and the competing ligands were added. The free [Mg^{2+}] was kept at 3 mM by addition of $MgCl_2$ and the ionic strength at 0.2 M by addition of KCl. Experiments on frog myofibrils were done at 0 °C, and the basic activating solution was 1 mM $MgCl_2$, 30 mM CaEGTA, and 100 mM *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES). The [Mg^{2+}] and ionic strength were kept at 1 mM and 0.2 M, respectively, as before. The same methods were used to measure the inhibition constants of the ligands on the ATPase activity of EDC-cross-linked acto-S1 and of acto-S1.

The contamination by ATP of ADP and AMPPNP was assayed by the luciferin/luciferase-linked assay system using an LKB luminometer and reagents. The average ATP contamination of AMPPNP was 0.1% and of ADP was 0.8%. In these experiments which lacked an ATP-regenerating system and in which diadenosine pentaphosphate is used to prevent myokinase activity, ADP contamination of AMPPNP is without effect. The ATP contamination was allowed for when making up the solutions. However, at the highest AMPPNP and ADP concentrations, the contaminant ATP provided almost all the ATP required, which reduced the accuracy with which the ATP concentration was known. At first sight, this might appear a significant source of error, but because of the nature of the assay, determination of the inhibition constant by this method is not critically dependent on the [ATP]. In the radioactive P_i assay, one is effectively measuring the time for complete hydrolysis of all ATP present (t_∞). If extra ATP is present, the ATPase rate is higher, but there is more ATP to be hydrolyzed: the effects thus tend to cancel out and do so completely for $[ATP] \ll K_m$. The effect is most easily seen by considering an example. Let the desired [ATP] be 5 μM but the actual ATP concentration in the presence of 4 mM AMPPNP be 10 μM. Let the K_m for ATP be 20 μM and K_{AMPPNP} be 4 mM. ATPase rate = ([ATP]/[myosin head])$(1/t_\infty)$. The ATP concentration is believed to be the same with and without the inhibitor, and thus for simplicity, we can set [ATP]/[myosin heads] = 1. The ATPase rate is now $1/t_\infty$, which is equal to $v/[ATP] = ([ATP]/\{K_m(1 + I/K_i) + [ATP]\})(1/[ATP]) = 1/\{K_m(1 + I/K_i) + [ATP]\}$. If [ATP] is 5 μM, the uninhibited ATPase rate is 1/25 = 0.04 and the inhibited ATPase rate is 1/45 = 0.022 (inhibition = 1 − 0.022/0.04 = 0.45). If in the inhibited case the ATP concentration is an fact 10 μM, then the measured ATPase rate is 1/50 = 0.02 (inhibition = 1 − 0.02/0.04 = 0.5). A 100% error in the [ATP] gives about a 10% error in the degree of inhibition.

Results

Rabbit Myofibrils. Figure 1 shows the time course of [^{32}P]ATP hydrolysis by rabbit myofibrils at a low concentration of MgATP (7 μM) and the inhibitory effect of increasing concentrations of ADP. The inhibitory effect is replotted as ATPase rate against ADP concentration in Figure 2 (▼), and it can be seen that a hyperbola is a satisfactory fit, consistent with the inhibition being competitive in nature. The

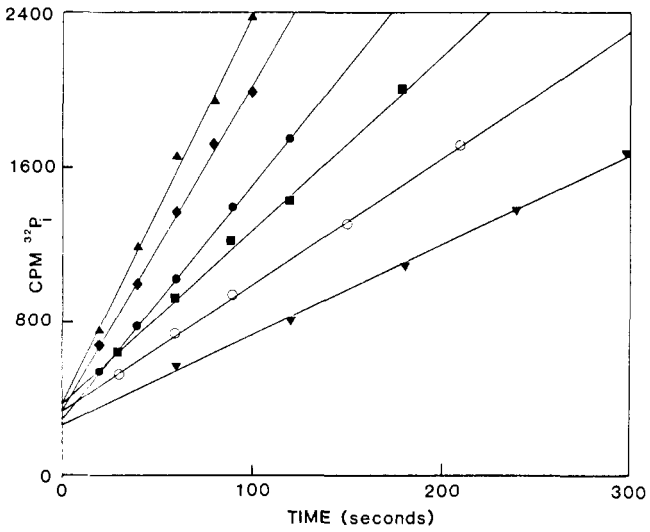


FIGURE 1: Rate of ATP hydrolysis by rabbit myofibrils at increasing concentrations of ADP. Basic reaction conditions were as follows: 15 °C, pH 7, 50 mM imidazole, 30 mM CaEGTA, 3 mM $MgCl_2$, 7 μM ATP, 0.05 mg/mL myofibrils. ADP was added at the stated concentrations, and $MgCl_2$ was added to conserve the free magnesium concentration at 3 mM and KCl to give an ionic strength of 0.2 M. [ADP]: (▲) 0, (♦) 75, (●) 150, (■) 300, (○) 500, and (▼) 1000 μM.

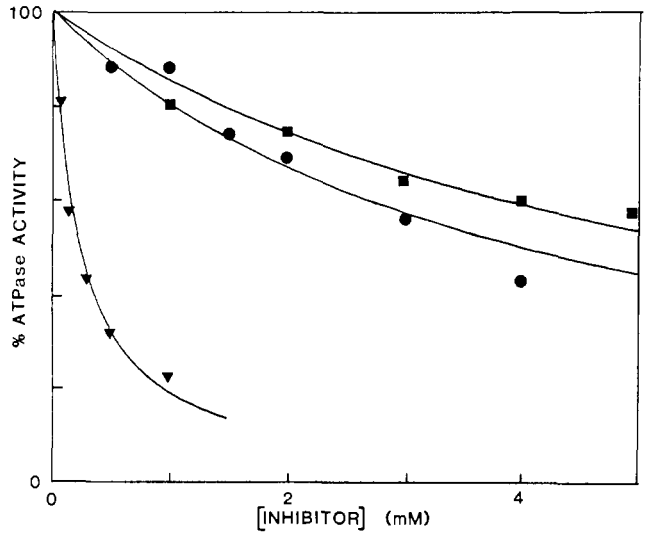


FIGURE 2: Inhibition of rabbit myofibrillar ATPase. ADP inhibition data (▼) from Figure 1; PP_i (●) and AMPPNP (■) inhibition data from similar experiments at 7 μM ATP. The curves correspond to apparent inhibition constants of 0.23, 3.9, and 5.8 mM, respectively.

fitted curve corresponds to a value for K'_{ADP} of 0.23 mM. To convert this to a true inhibition constant, K_{ADP}, the value of K_m is needed. This was obtained from a series of experiments of the type shown in Figure 1; a secondary plot of rate vs. [ATP] for the same preparation of myofibrils is shown in Figure 3. The value of K_m is 21 μM, and thus K_{ADP} is 0.23/(1 + 7/21) = 0.17 mM. Included in Figure 2 are plots of the inhibitory effect of AMPPNP (■) and PP_i (●) on the same myofibril preparation. The values of K'_{PP_i} and K'_{AMPPNP} are 3.9 and 5.8 mM, respectively, which give inhibition constants of 2.9 and 4.3 mM, respectively. Hyperbolas have been fitted to the inhibition data of all three ligands, but the extent of inhibition of PP_i and AMPPNP is not sufficient to demonstrate a hyperbolic dependence.

The first efforts at measuring the K_m for ATP from the initial rates were rather variable. The ATP dependence often looked biphasic, and if, despite the appearance, the data were fitted to a hyperbola, the resultant K_m was around twice that

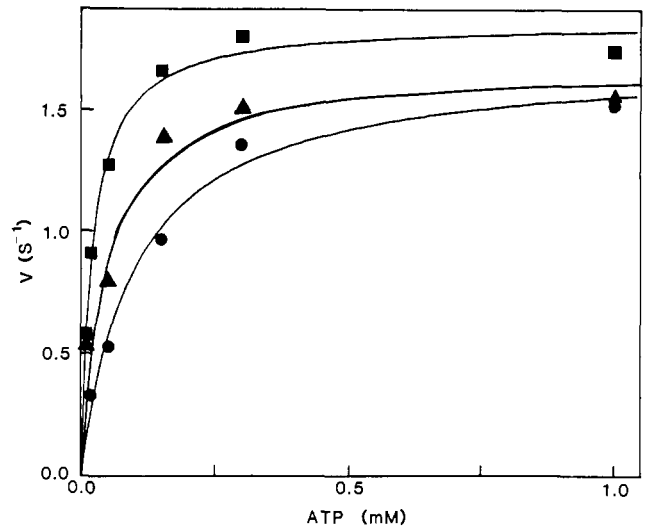


FIGURE 3: Effect of ADP concentration on the apparent K_m' for ATP. Reaction conditions as for Figure 1. (■) No added ADP, K_m = 21 μM; (●) in the presence of 0.8 mM ADP, K_m' = 99 μM; (▲) in the presence of 5 mM AMPPNP, K_m' = 41 μM.

observed by using a linked assay. The biphasicity suggested that this was due to an inhomogeneous population of myofibrils, single myofibrils giving a low K_m and clumped myofibrils giving a high K_m, the value of V_m being satisfactory. By allowing dilute suspensions of myofibrils to settle for about 2 h prior to taking the superntant, the homogeneity of the preparation was considerably improved, and when this procedure was carried out both before and after cross-linking, satisfactory plots of the type shown in Figure 3 were obtained. For each cross-linked preparation, an initial measurement of the K_m for ATP was made, and the occasional preparation for which the value was more than 30 μM was rejected. In the case of the linked assay system, it is less critical that the myofibrils are uniformly single because the problem of diffusion of nucleotide into the myofibrils is much less severe due to the linking enzymes regenerating much of the ATP within the myofibril. These problems were more serious for frog myofibrils, consistent with their lower degree of homogeneity.

The inhibitory effect of the ligands can also be determined by comparison of the apparent value of the K_m for ATP in the presence of a constant concentration of ligand with the true value of K_m in the absence of the competing ligand. An example of this method is shown in Figure 3. The apparent K_m' in the presence of 0.8 mM ADP is 99 μM. This value in association with the true K_m in the absence of ligand, 21 μM, gives an inhibition constant of 0.22 mM. In the presence of 5 mM AMPPNP, the value of K_m' is 41 μM which gives a K_i of 5.3 mM. The values for K_i determined in this manner were not significantly different from those obtained by increasing the ligand concentration although it should be noted that for both methods the errors in the determination for AMPPNP and PP_i are quite large due to the weak inhibition. The average inhibition constants for a number of measurements using both methods for rabbit myofibrils are 0.17 ± 0.05 mM (mean ± SD; n = 7), 3.0 ± 1.5 mM (n = 8), and 5.0 ± 2 (mM (n = 5) for ADP, PP_i, and AMPPNP, respectively.

As a check on the measurement of inhibition constants by the limited hydrolysis of [^{32}P]ATP, the inhibition of rabbit myofibrillar ATPase was also checked by using a linked assay in a spectrophotometer. The experiment was done both by measuring the K_m for ATP in the absence and presence of PP_i (2 and 3 mM) and also by measuring the inhibition at increasing PP_i concentrations at a MgATP concentration of 20

μM. The first method gave a value of 2.2 ± 0.8 mM (n = 5), and two measurements with the second method gave values of 1.65 and 2.25 mM: both results are in reasonable agreement with those obtained by using [^{32}P]ATP.

At a concentration of free Mg^{2+} ions of 3 mM, only 56% of the ADP is in the form of MgADP, and the question arises as to whether ADP as well as MgADP is an inhibiting species. For this reason, the inhibitory effect of 0.5 mM total ADP on the hydrolysis of 7 μM ATP was measured in the presence of 0.5 and 5 mM free Mg^{2+}, which resulted in 18% and 61%, respectively, of the ADP being in the form of the magnesium complex. The extent of inhibition was 40% and 36%, respectively: the difference is hardly outside experimental error. ADP is thus as good or almost as good an inhibitor as MgADP. Equivalent observations have been made on the similarity both of the binding of ADP and MgADP to fibers (Marston, 1973) and of their effect on shortening the velocity (M. Iino, J. Rondinone, and R. M. Simmons, unpublished results).

The formation of ATP by the action of myokinase is a potential problem, particularly in the case of ADP inhibition experiments. Diadenosine pentaphosphate was added to a concentration of 5 μM or 10% of the ADP concentration, whichever was the greater in all experiments. The effective inhibition of myokinase activity was checked with the pH stat. The rate of H^+ release on addition of 2 mM MgADP to a suspension of myofibrils was too slow to measure (less than 5% of the rate given by 1 mM MgATP). When exogenous myokinase was added, a high rate developed which was almost totally inhibited at the concentrations of diadenosine pentaphosphate used. In an experiment in which myofibrils were inhibited by 1 mM ADP, it was found that in the absence of diadenosine pentaphosphate inhibition was 20% more severe, showing that its inclusion is important and yet not so critical that the experiment stands or falls upon its efficacy.

Inhibition of Frog Myofibrillar ATPase. Similar experiments were carried out for frog myofibrils with similar results. The inhibition constants were as follows: ADP, 0.22 ± 0.06 mM (n = 7); PP_i, 1.4 ± 1.0 mM (n = 4); AMPPNP, 2.5 ± 1.2 mM (n = 4).

Inhibition of Acto-S1 and EDC-Cross-Linked Acto-S1. We were particularly interested in comparing the inhibition of acto-S1 with that of myofibrils, but actin gives negligible activation of S1 ATPase at the ionic strength of the myofibril experiments (0.2 M). For this reason, we used EDC-cross-linked acto-S1, a preparation which appears to be fully actin activated even at high ionic strength. The value of the K_m for ATP measured in the same manner as described for myofibrils in Figure 3 was 15 μM. The inhibition constants under these conditions are 0.12 ± 0.05 mM (n = 5), 2.6 ± 0.5 mM (n = 6), and 3.5 ± 1 mM (n = 5), respectively. This approach suffers from the possible criticism that EDC-cross-linked acto-S1 may not be representative in all respects of acto-S1 at saturating actin concentration, and for this reason, the inhibition of cross-linked acto-S1 was compared to native acto-S1 at low ionic strength (0.03 M). A fairly high actin concentration (30 μM) was used so that the ATPase was almost fully activated. The properties of the two preparations were very similar. The linked assay system was used to check the inhibition by PP_i of EDC-cross-linked acto-S1 at ionic strengths of 0.2 and 0.03 M and of actin-activated S1 at an ionic strength of 0.03 M. The results were in reasonably good agreement with those from the radioactive assay.

Discussion

Values were obtained in the present work for the inhibition by ligands of the ATPase of acto-S1 and of myofibrils. These must be compared with the corresponding values for the binding constants. The three basic questions raised are as follows: Should the acto-S1 dissociation constant equal the acto-S1 inhibition constant, should the fiber dissociation constant equal the acto-S1 dissociation constant, and should the myofibril inhibition constant equal the acto-S1 dissociation constant? Results of measurements of these constants from several sources have been brought together for comparison in Table I.

Table I: Comparison of Inhibition and Dissociation Constants

	ADP	PP_i	AMPPNP
Inhibition Constants (mM) from ATPase			
rabbit myofibrils	0.17 ± 0.05 (n = 7)	3.0 ± 1.5 (n = 8)	5.0 ± 2.0 (n = 5)
frog myofibrils	0.22 ± 0.06 (n = 7)	1.5 ± 1.0 (n = 4)	2.5 ± 1.2 (n = 4)
rabbit acto-S1	0.12 ± 0.05 (n = 5)	2.6 ± 0.5 (n = 6)	3.5 ± 1.0 (n = 5)
Reported Inhibition Constants (mM) from Shortening Velocity			
rabbit	0.3[a]	3.0[a]	5.0[a]
frog			4.0[b]
Reported Binding Constants (mM)			
rabbit myofibrils	0.17[c]		
rabbit fibers	0.035[d]		0.03[e]
acto-S1	0.14[f]	0.2[f]	0.33[f]
			0.12[g]
	0.14[h]	2.0[h]	2.0[h]

[a] Pate & Cooke (1984). [b] M. Iino, J. Rondinone, and R. M. Simmons (unpublished results). [c] Johnson & Adams (1984). [d] Marston (1973). [e] Marston et al. (1976). [f] Greene & Eisenberg (1980). [g] Konrad & Goody (1982). [h] Biosca et al. (1985).

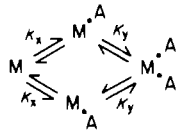
Acto-S1 Inhibition Constant vs. Dissociation Constant. The acto-S1 ATPase inhibition constants for ADP, PP_i, and AMPPNP are 0.12, 2.6, and 3.5 mM, respectively. The ADP inhibition constant was in good agreement with the dissociation constant (0.14 mM; Greene & Eisenberg, 1980), but the PP_i and AMPPNP inhibition constants were in poor agreement with the dissociation constants (0.2 and 0.15–0.33 mM; Greene & Eisenberg, 1980; Konrad & Goody, 1982). For an inhibitor which binds to the same rigor actomyosin complex as ATP, the inhibition constant must be the same as the dissociation constant. The introduction into the scheme of extra nucleotide-free states which could bind the inhibitor does not allow the inhibition constant to be weaker than the dissociation constant. Either the dissociation constant or the inhibition constant must be wrong.

In a recent abstract, Biosca et al. (1985) reported a remeasurement of the dissociation constants of ADP, PP_i, and AMPPNP. In the case of ADP, they confirmed the original measurement, but in the case of PP_i and AMPPNP, they found the original measurement to be in error. Both PP_i and AMPPNP bind much more weakly than previously reported, the dissociation constants being about 2 mM. The reason for the error may have been that the original measurements on ligand binding to acto-S1 were done at actin concentrations that allowed part of the S1 to be dissociated from actin and thus display a tight binding constant. The ligand dissociation constant deduced from these measurements is very sensitive to the fraction of S1 with actin bound. Final resolution of this problem will have to await a full report of this work.

Fiber and Myofibrillar Dissociation Constant vs. Acto-S1 Dissociation Constant. We will accept the revised results of Greene and collaborators and thus use 0.14, 2, and 2 mM for the acto-S1 dissociation constants of ADP, PP_i, and AMPPNP, respectively. The dissociation constants of fibers for ADP and AMPPNP are 0.03 (Marston, 1973) and 0.035

mM (Marston et al., 1976), respectively. The dissociation constant of myofibrils for ADP is 0.17 mM (Johnson & Adams, 1984). Myofibrils would be expected to behave the same as fibers. Johnson and Adams suggested that the disrepancy might be due to myokinase activity in fibers resulting in a measurement which in the limit would be the K_m for ATP, but Marston had considered this possibility and deduced that it was not a factor.

The differences between the inhibition and dissociation constants of AMPPNP and PP_i are large and must be due either to a systematic error in one of the measurements or to cooperativity between myosin heads. Further work may demonstrate such errors, but in the meantime, it is appropriate to consider cooperative models. If in a fiber one head of each



myosin molecule is dissociated in the presence of AMPPNP or PP_i, then the binding constant of the dissociated head will become closer to that of free myosin (M). There is evidence from a variety of techniques that a substantial proportion of heads are dissociated or weakly bound in the presence of AMPPNP (Thomas & Cooke, 1980; Yanagida, 1981; Chen & Reisler, 1984). The effective actin concentration of a muscle appears to be a few millimolar (Brenner et al., 1983), and the binding constant of actin to M·AMPPNP is 0.06 mM (Greene & Eisenberg, 1980), and thus one would certainly expect at least one head of each myosin (M) to be bound. The possibility of the second head being dissociated is apparent from the deduction of Greene and Eisenberg (1980b) that the equilibrium constant of the binding of the second head of heavy meromyosin (HMM) to actin is of the order of 1000 and the observation that AMPPNP weakens actin binding by a factor about 600 (Greene & Eisenberg, 1980a). It can also be argued that the observation that the stiffness of a fiber in the presence of AMPPNP is the same as in rigor (Marston et al., 1979) indicates that S2 is the compliant element of the cross bridge and that at least one head of each myosin remains bound in the presence of AMPPNP. If 50% of the heads were dissociated and there was no cooperativity between head binding, the distribution of myosin molecules with no heads, one head, and both heads bound would be 25%, 50%, and 25%, respectively. The dependence of the proportion of myosin molecules with zero, one, and two heads bound can readily be derived. For 50% of the heads to be dissociated, $K_xK_y = 1$. If the degree of negative cooperativity is a factor of 10 ($K_y = K_x/10$), then 77% of myosin molecules would have one head bound. A lack of cooperativity would represent a fortuitous balance of the two competing forces of entropy leading to positive cooperativity and steric strain leading to negative cooperativity.

The predicted binding behavior if one head is dissociated depends on the kinetics of interaction of actin and AMPPNP with S1. If actin binding equilibrates faster than AMPPNP binding, then the binding behavior of free and actin-bound heads will average to be described as a single binding constant. The rates of AMPPNP dissociation from acto-S1 and S1 are about 500 and 0.02 s^{-1}, respectively, and upon dilution, actin dissociates from AM·AMPPNP at about 1 s^{-1} (Konrad & Goody, 1982). If a myosin has one head bound to actin and one head free, then after about a second, the bound head dissociates, and the heads become equivalent so that the formerly dissociated head is equally likely to bind to actin. Half the heads would be predicted to display a considerably weakened form of the AMPPNP binding behavior of myosin. However, as AMPPNP dissociates from AM·AMPPNP very much faster (500 s^{-1}) than actin dissociates (1 s^{-1}), half the heads would be predicted to exhibit the binding behavior of acto-S1. The AMPPNP binding of the dissociated heads is weakened by the occasional binding to myosin whereas the binding of associated heads is unaffected by occasional dissociation.

The interaction of insect fibers with AMPPNP is of particular interest because in rigor about 25% of the heads are not bound to actin (Goody et al., 1985) and would be definitely predicted to display tight binding. The AMPPNP binding data for insect fiber (Marston et al., 1976) could be satisfactorily fitted by two dissociation constants, but the low one would only account for about 25% of the total binding. The low dissociation constant is much larger than that of myosin, consistent with the kinetic predictions above. Reedy et al. (1983) have suggested on the basis of X-ray and electron microscopy studies of insect muscles that AMPPNP binding leads to a state in which all myosin molecules have one head bound to actin and one head free.

In a recent abstract, Johnson (1985) reported that either myofibrils display two classes of AMPPNP binding sites or AMPPNP binds with negative cooperativity. In the case of ADP, due to its relatively poor dissociating effect, the heads remain bound, and thus the binding remains homogeneous and characteristic of acto-S1 in agreement with the data of Johnson and Adams (1984) on the binding of ADP to myofibrils. The only data that appear inconsistent with interpreting the effect of AMPPNP on fibers in terms of dissociation of one of the myosin heads are that the stoichiometry of AMPPNP binding to insect fibers is similar both to that of ADP and to the estimate of the concentration of myosin heads in rabbit muscle myofibrils (Yates & Greaser, 1983).

If the tight binding of AMPPNP to fibers does result from two classes of myosin heads, then the possibility is raised that the apparent chemomechanical effect of AMPPNP binding is due to dissociation. The most strained cross bridges are released on AMPPNP binding, resulting in a reduction of tension; conversely, stretching a fiber strains the cross bridges, leading to dissociation and the exhibition of tight binding (Marston et al., 1979). For technical reasons, the enhancement of AMPPNP binding on stretching a muscle was done at a low [AMPPNP] at which less than 30% of the heads would have bound AMPPNP. As already discussed, the high rate of AMPPNP dissociation means that not all the heads can be equivalent and thus the heads under investigation must have been those interacting only occasionally with actin.

Myofibril ATPase Inhibition vs. Acto-S1 Dissociation Constant. The ADP inhibition constant of rabbit myofibrillar ATPase is 0.17 mM, and this is very similar to the reported dissociation constant of ADP to acto-S1 (0.14 mM; Greene & Eisenberg, 1980). The PP_i and AMPPNP inhibition constants of rabbit myofibrillar ATPase are 3 and 5 mM, respectively, which are acceptably close to the revised acto-S1 dissociation constants which are both about 2 mM (Biosca et al., 1985). The similarity of the inhibition constants of cross-linked myofibrillar and of acto-S1 ATPase implies that the combination of any strain induced during contraction and any chemomechanical effect do not combine to change significantly the binding properties of the rigor heads in an isometric fiber from those of acto-S1. This is to be expected because the chemomechanical effect is quite small, maximal stretching of a muscle resulting in halving of the dissociation

constant (Marston et al., 1979). When a muscle fiber is shortening, any rigor heads must experience a negative tension, and the similarity of the inhibition constants describing shortening velocity (Pate & Cooke, 1984) with those describing acto-S1 and myofibrillar ATPase is again consistent with the small size of any chemomechanical effect.

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Registry No. ADP, 58-64-0; PP_i, 14000-31-8; AMPPNP, 25612-73-1; ATPase, 9000-83-3; ATP, 56-65-5.

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Effect of Muscle Tropomyosin on the Kinetics of Polymerization of Muscle Actin†

Altaf A. Lal‡ and Edward D. Korn*

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT: At saturating concentrations, tropomyosin inhibited the rate of spontaneous polymerization of ATP-actin and also inhibited by 40% the rates of association and dissociation of actin monomers to and from filaments. However, tropomyosin had no effect on the critical concentrations of ATP-actin or ADP-actin. The tropomyosin–troponin complex, with or without Ca^{2+}, had a similar effect as tropomyosin alone on the rate of polymerization of ATP-actin. Although tropomyosin binds to F-actin and not to G-actin, the absence of an effect on the actin critical concentration is probably explicable in terms of the highly cooperative nature of the binding of tropomyosin to F-actin and its very low affinity for a single F-actin subunit relative to the affinity of one actin subunit for another in F-actin.

Polymerization of actin has two major phases: the relatively slow formation of nuclei, most probably trimers, and the more rapid elongation of nuclei to long filaments by addition of monomers to each end (Korn, 1982). The details of the nucleation process are still rather obscure, but considerable information is now available about the elongation phase. Most simply, the kinetics of elongation can be described by the equation

$$dF/dt = k_+Nc_1 - k_-N = k_+N(c_1 - c_c) \quad (1)$$

where k_+ and k_- are the sums of the association and dissociation rate constants at the barbed and pointed ends[1] of the filaments, N is the filament number concentration, c_1 is the actin monomer concentration, and c_c is the critical concentration = k_-/k_+.

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* Address correspondence to this author.

‡ Permanent address: National Institute of Immunology, New Delhi, 110029 India.

[1] The ends of actin filaments are designated "barbed" and "pointed" from the appearance in the electron microscope of filaments decorated with muscle heavy meromyosin.