

ADP Binding to Myosin Cross-Bridges and Its Effect on the Cross-Bridge Detachment Rate Constants

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ABSTRACT We have studied the binding of adenosine diphosphate (ADP) to attached cross-bridges in chemically skinned rabbit psoas muscle fibers and the effect of that binding on the cross-bridge detachment rate constants. Cross-bridges with ADP bound to the active site behave very similarly to cross-bridges without any nucleotide at the active site. First, fiber stiffness is the same as in rigor, which presumably implies that, as in rigor, all the cross-bridges are attached. Second, the cross-bridge detachment rate constants in the presence of ADP, measured from the rate of decay of the force induced by a small stretch, are, over a time scale of minutes, similar to those seen in rigor. Because ADP binding to the active site does not cause an increase in the cross-bridge detachment rate constants, whereas binding of nucleotide analogues such as adenylyl-5'-yl imidodiphosphate (AMP-PNP) and pyrophosphate (PP_i) do, it was possible, by using ADP as a competitive inhibitor of PP_i or AMP-PNP, to measure the competitive inhibition constant and thereby the dissociation constant for ADP binding to attached cross-bridges. We found that adding 175 μ M ADP to 4 mM PP_i or 4 mM AMP-PNP produces as much of a decrease in the apparent cross-bridge detachment rate constants as reducing the analogue concentration from 4 to 1 mM. This suggests that ADP is binding to attached cross-bridges with a dissociation constant of $\sim 60 \mu$ M. This value is quite similar to that reported for ADP binding to actomyosin subfragment-1 (acto-S1) in solution, which provides further support for the idea that nucleotides and nucleotide analogues seem to bind about as strongly to attached cross-bridges in fibers as to acto-S1 in solution (Johnson, R. E., and P. H. Adams. 1984. *FEBS Letters*. 174:11–14; Schoenberg, M., and E. Eisenberg. 1985. *Biophysical Journal*. 48:863–871; Biosca, J. A., L. E. Greene, and E. Eisenberg. 1986. *Journal of Biological Chemistry*. 261:9793–9800).

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INTRODUCTION

In fibers, as in solution, ATP and ATP analogues such as adenylyl-5'-yl imidodiphosphate (AMP-PNP) and pyrophosphate (PP_i) bind to attached cross-bridges, causing a large increase in the cross-bridge detachment rate constants. An important step in better understanding cross-bridge behavior is to determine how the interaction between cross-bridges and actin in fibers relates to the biochemistry of actin and myosin in solution. Two ways of approaching this are to examine and relate the various rate constants in the fiber to those in solution and to compare the strength of binding of different nucleotides or nucleotide analogues in fibers and in solution.

Previously (Schoenberg and Eisenberg, 1985; Brenner et al., 1986a; Schoenberg, 1987), we examined the cross-bridge detachment rate constants in fibers, in the presence of ATP and also AMP-PNP and PP_i . We found that, in the presence of ATP, in relaxed fibers, cross-bridges detach exceedingly rapidly, with rate constants $>10^3 \text{ s}^{-1}$ (Brenner et al., 1986a; Schoenberg, 1987). In solution also, the detachment rate constant of subfragment-1·ATP (S1·ATP) from actin is rapid; the rate constant is reported to be $>10^3 \text{ s}^{-1}$ (Lymn and Taylor, 1971; Millar and Geeves, 1983). Although these rate constants are similar, the precise relation between the fiber and solution rate constants is unclear because, in both cases, the measured rate constants are near the limit of the instrumentation.

In the presence of AMP-PNP or PP_i , the detachment rate constants can also be compared in the fiber and in solution; here, the rate constants are somewhat slower, so that precise measurements can be made. For the fiber, a rather wide range of cross-bridge detachment rate constants is found (Schoenberg and Eisenberg, 1985), the fastest of which are comparable to the detachment rate constant of S1 from actin in solution (Marston, 1982; Konrad and Goody, 1982). The slowest detachment rate constants in the fiber are considerably slower than the S1 detachment rate constant, but the reason for this is not currently known (Tozeren and Schoenberg, 1986).

We also found that these analogues bind rather weakly to attached cross-bridges. At the time we obtained this result, most biochemical studies (Hofmann and Goody, 1978; Greene and Eisenberg, 1980; Konrad and Goody, 1982), as well as fiber studies (Marston et al., 1976; Goody et al., 1976), suggested that the binding of AMP-PNP either to actomyosin subfragment-1 (acto-S1) in solution or to attached cross-bridges in fibers was strong. However, more recent studies, both in fibers (Pate and Cooke, 1985) and in solution (Johnson, 1986; Biosca et al., 1986), support our finding that AMP-PNP and PP_i bind weakly, both in fibers and in solution.

Because of the disagreement about the tightness of binding of AMP-PNP and PP_i , we looked at the behavior of another nucleotide for which tight binding had been reported: ADP. This gave us the opportunity to examine, in another instance, the relationship between the solution and fiber binding constants and also the solution and fiber detachment rate constants. We found that ADP binds tightly to attached cross-bridges in fibers, just as it does to acto-S1 in solution, and we also found that, just as for AMP-PNP and PP_i , the binding of ADP at

the active site results in fiber detachment rate constants significantly slower than those for S1 detachment from actin in solution. Furthermore, somewhat surprisingly, in the fiber, the apparent detachment rate constants in the presence of ADP were not significantly different from those seen in rigor.

METHODS

Preparation, Mounting, and Solutions

The procedure for the preparation and mounting of freshly skinned rabbit psoas fibers was similar to that described previously (Schoenberg and Eisenberg, 1985), with the exceptions that (a) fibers were not always studied immediately after dissection but were often left refrigerated in dissecting solution overnight, and (b) the solutions were changed slightly to reduce the amount of Cl and increase the buffering of Ca^{2+} and ATP (see Table I). The dissecting solution, similar to that of Eastwood et al. (1979), was identical to that used previously and contained 150 mM K-propionate, 3 mM Mg-acetate, 3 mM

TABLE I
Composition of Solutions

Solution	K-propionate	Imidazole	EGTA or CaEGTA*	EDTA	Excess Mg^{2+}	Mg-nucleotide or Mg-analogue	Phospho-creatine	DTT	Ionic strength
				<i>mmol/liter</i>					
Relaxing	135	10	3	—	2	3	—	0.5	170
Contracting	100	10	5*	—	2	3	10	0.5	170
Quick rinse	80	20	5	15	—	—	—	—	150
Rigor	90	10	3	—	2	—	—	0.5	110
4 mM Mg-analogue	70	10	3	—	2	4	—	0.5	110
1 mM MgADP	87	10	3	—	2	1	—	0.5	110

pH 7.0 ± 0.05 at 5°C .

Na_2ATP , 5 mM EGTA, 0.5 mM dithiothreitol (DTT), and 5 mM KH_2PO_4 , pH 6.8 at 5°C .

All reagents were analytic grade or purer. The EGTA and imidazole were obtained from Eastman Kodak Co., Rochester, NY. Na_2ATP (A-5394), $\text{Li}_4\text{AMP-PNP}$ (A-2647), the K (A-5410) or Li (A-4907) salt of ADP, *p,p*-di(adenosine-5')pentaphosphate (Ap_5A) (D-6392), hexokinase (H-5875), and DTT were all obtained from Sigma Chemical Co., St. Louis, MO. Before use, the hexokinase, a precipitate in ammonium sulfate, was spun down and resuspended in rigor solution (Table I).

Mechanical Procedures

The equipment was identical to that used in Schoenberg and Eisenberg (1985). The sarcomere length was initially set to $2.5 \mu\text{m}$ and all mechanical perturbations were done by controlling the sarcomere length using the sarcomere-length detector-follower previously described. This avoided artifacts caused by end compliance of the glued fibers. At the end of each experiment, the maximum isometric tension, P_0 , was measured at 5°C in contracting solution (Table I).

Solution Changes and Induction of Rigor

Rigor was induced as in Schoenberg and Eisenberg (1985), using the quick-rinse and rigor solutions listed in Table I. Basically, the fiber was first cooled to near 0°C , then cold

quick-rinse (EDTA-containing) solution was washed through the 2.5-ml chamber, and only after ≥ 10 chamber volumes had been exchanged was the quick-rinse solution replaced with rigor solution. This procedure induced rigor quickly, producing a fiber that had shortened very little during rigor induction ($<0.5\%$), exerted little tension, and had a rather uniform striation pattern. Fibers were always put into rigor before going into PP_i -, AMP-PNP-, or ADP-containing solutions. All experiments were performed at $5 \pm 1^\circ\text{C}$.

Measurement of Force Decay Half-Times

The force decay data were collected, signal-averaged, and digitized using a digital processing oscilloscope (model 4094, Nicolet Instrument Corp., Madison, WI). They were then transmitted via an RS-232 interface to a 68010-based supermicrocomputer (MC-500, Masscomp, Littleton, MA). The half-times for tension decay were obtained by first displaying the decay of force, plotted against the logarithm of time after start of stretch, on a Concept GVT graphics terminal (Human Design Systems Inc., Philadelphia, PA). Using cross-hairs, the initial tension (the tension 1–3 ms after the start of stretch) was estimated. Then, again using cross-hairs, the time for the tension to decay 50% was measured by eye.

Controls for ATP Contamination

Previously (Schoenberg and Eisenberg, 1985), we showed that when the ends of a rigor fiber are released so that the tension of the fiber is reduced to just slightly above zero, little tension redevelopment occurs unless ATP is present. We also showed that as little as $1 \mu\text{M}$ ATP causes a significant increase in the rate and amount of tension redevelopment. By this criterion, a solution made up from rigor solution plus 1 mM commercially available MgADP often showed significant amounts of contaminant ATP when added to muscle fibers. Two possible sources of the contaminant ATP are the ATP produced from ADP by the fiber myokinase and the contaminant ATP present in the ADP stock solution.

To reduce the contaminant ATP caused by myokinase activity, $225 \mu\text{M}$ Ap_5A was added to all bath solutions. The contaminant ATP in the stock ADP was reduced either by including 2 mM glucose and 10 Sigma U/ml of hexokinase in the bath solutions or by a procedure in which 10 ml of 80 mM stock ADP was incubated overnight, at 5°C , in a solution (pH 7.0) containing 0.8 ml of 1 M MgCl_2 , 0.04 ml of 0.5 M imidazole, and 1.5 ml of $15 \mu\text{M}$ cross-linked acto-S1 prepared according to the procedure of Mornet et al. (1981). After overnight incubation, the cross-linked acto-S1 and any free actin were removed by first centrifuging the ADP-protein mixture for 1 h at 45,000 rpm and then centrifuging it for 2 h at 5,000 rpm through a Centricon filter (Amicon Corp., Danvers, MA). The final concentration of MgADP was determined spectrophotometrically.

On the basis of the force redevelopment assay, the fibers appeared to be quite variable with regard to their apparent myokinase activity. Thus, Fig. 1A shows the force redevelopment from a fiber showing an unusually large amount of tension redevelopment in the presence of 1 mM untreated ADP in the absence of Ap_5A . High concentrations of Ap_5A reduced but did not eliminate the force redevelopment after release. 400 and 800 μM Ap_5A gave the same response, which suggests that the residual tension redevelopment was due directly to ATP contamination. The finding that the addition of glucose and hexokinase further inhibited the force redevelopment after release supported this idea (data not shown). Fig. 1B shows data from a somewhat more typical fiber, which had a much smaller force redevelopment response in 1 mM untreated ADP without Ap_5A .

Our impression was that the Ap_5A -sensitive component of force redevelopment decayed on a time scale of tens of minutes after the skinning of the fiber. Two fibers that were

stored overnight in dissecting solution showed no force redevelopment when placed in a solution that had no Ap_5A but contained ADP previously incubated with cross-linked acto-S1. This suggests that much of the myokinase activity may, in time, diffuse out of the fiber, although this was not studied in a careful way.

Aside from the above-mentioned controls, all experiments were done in the presence of $225 \mu\text{M}$ Ap_5A . Most experiments were done with ADP preincubated with cross-linked acto-S1, but several were performed using unincubated ADP, with 2 mM glucose and 10 U/ml hexokinase added to the bathing solutions. About three-fourths of the experiments were done on fibers stored overnight; the remainder were done on freshly dissected fibers. The possibility exists that the state of myosin light-chain phosphorylation was different in the fibers stored overnight (Persecchini et al., 1985). Nevertheless, none of the differences in experimental protocol seemed to influence the results, and with each of the above procedures, all fibers tested showed just about the same force redevelopment in the presence of 1 mM ADP as in normal rigor solution.

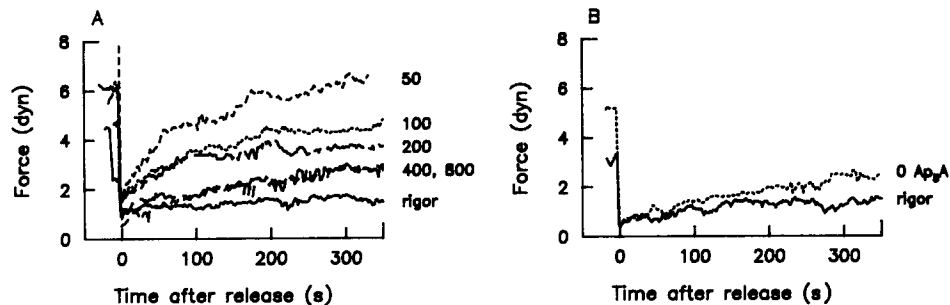


FIGURE 1. (A) Force redevelopment as a function of Ap_5A concentration after a quick release to a near-zero load of a freshly dissected rabbit psoas fiber in rigor solution, or in a solution made up of rigor solution plus 1 mM untreated MgADP. The concentrations of Ap_5A are given in micromolar next to each curve. In the absence of Ap_5A , the force redevelopment was >15 dyn (not shown). This fiber showed an unusually large force redevelopment response and an unusually large response to Ap_5A . (B) Fiber showing a much smaller, more typical amount of tension development in 1 mM MgADP. Experiments on both fibers were done with unincubated ADP, without added glucose or hexokinase. (A) Experiment 010785. Diameter, $115 \times 105 \mu\text{m}$. $P_0 = 75$ dyn. (B) Experiment 010985. Diameter, $58 \times 125 \mu\text{m}$. $P_0 > 40$ dyn.

RESULTS

Addition of ADP to Rigor Fibers

Recently, Kawai (1986) reported that the addition of MgADP to a fiber in rigor leads to a significant increase in force. Whereas we often saw, even in the presence of $225 \mu\text{M}$ Ap_5A , a rise in force in fibers exposed to untreated MgADP, we never saw this rise when the MgADP was preincubated with glucose and hexokinase or cross-linked acto-S1 (see Methods). Instead, the addition to rigor fibers of MgADP treated to remove contaminant ATP always resulted in a small but definite decrease in force (Marston et al., 1976).

Cross-Bridge Detachment Rate Constants in the Presence of ADP

Previously (Schoenberg, 1985), we showed that the decay rate of the force generated by a small stretch applied to a muscle fiber provides information about the cross-bridge detachment rate constants. When a quick stretch of 2 nm per half-sarcomere is applied to a single skinned rabbit psoas fiber in rigor or in 1 mM ADP solution, a force of 15–25 dyn is induced simultaneously with the stretch. Fig. 2 shows the subsequent decay of that force with the fiber held isometric in each of the two solutions. The first thing to note is that the stiffness in 1 mM ADP solution is the same as that in rigor. In addition, it is seen that, over a period of 300 s, the tension decay in the presence of 1 mM ADP is also not significantly different from that in rigor. Either the dissociation constant (K_d) for ADP binding to the fibers is so large that a significant amount of ADP is not

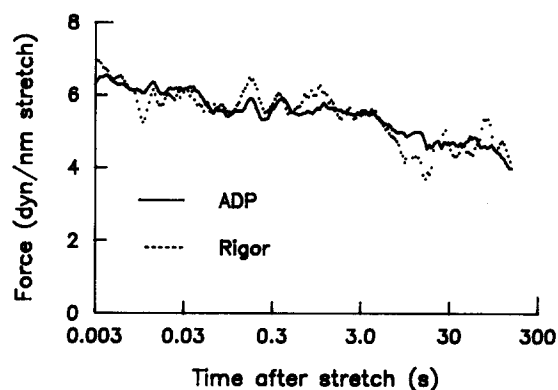


FIGURE 2. Force relaxation after stretch in rigor and in the presence of 1 mM ADP, on a time scale of minutes. Note that the force decay in rigor is small and that in 1 mM ADP is not significantly different. The stretch was nominally 2 nm per half-sarcomere. The experiment was done with preincubated ADP and 225 μ M A_p_5A . Experiment 010786. Diameter, $50 \times 82 \mu$ m. $P_0 = 46$ dyn.

binding at a concentration of 1 mM, or the binding of ADP does not significantly increase the cross-bridge detachment rate constants relative to those in rigor (at least over a 5-min period). As shown below, the latter conclusion is the correct one.

The Strength of Binding of ADP to Attached Cross-Bridges

One way of determining whether 1 mM ADP is binding to attached cross-bridges is by competing ADP with either PP_i or AMP-PNP. Previously (Schoenberg and Eisenberg, 1985), we showed that adding PP_i or AMP-PNP to a rigor fiber greatly increases the rate of force decay after a stretch. In contrast, as Fig. 2 shows, on a time scale of minutes, the force decay in the presence of 1 mM ADP is no more rapid than it is in the absence of nucleotide. This means that if 1 mM ADP is binding to attached cross-bridges, ADP should be a potent inhibitor of the PP_i effect. As Fig. 3A shows, ADP is indeed a very good inhibitor of the PP_i effect. A comparison of panels A and B of Fig. 3 shows that when concentrations of ADP as small as 100–200 μ M are added to 4 mM PP_i , this produces as big an

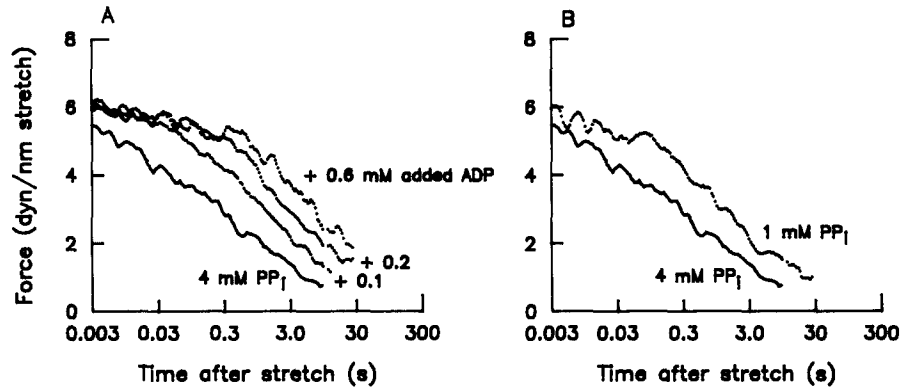


FIGURE 3. Inhibition of force relaxation after stretch by either addition of ADP (A) or reduction of the MgPP_i concentration from 4 to 1 mM (B). The stretch was nominally 2 nm per half-sarcomere. The sarcomere length was held constant after stretch by means of a sarcomere-length detector-follower operated with feedback. Note that 0.1–0.2 mM MgADP added to 4 mM MgPP_i retards the force relaxation as much as reducing MgPP_i from 4 to 1 mM. The K_i calculated from these data was $33 \mu\text{M}$. Experiment 112685. Diameter, $73 \times 82 \mu\text{m}$. $P_0 = 56 \text{ dyn}$.

effect as reducing the concentration of PP_i from 4 to 1 mM. This suggests that ADP and PP_i are binding competitively to the active site of myosin, with ADP binding considerably more tightly. Fig. 4 shows that the same is true with ADP and AMP-PNP. Here again, adding $200 \mu\text{M}$ ADP produces about as big an effect as reducing the concentration of AMP-PNP from 4 to 1 mM; adding $2,000 \mu\text{M}$ ADP produces a much bigger effect.

It is possible to obtain directly, from either Fig. 3 or 4, the competitive inhibition constant (K_i) for ADP, which is presumably equal to the K_d for ADP binding. The usual way of analyzing a competition experiment is to assume that

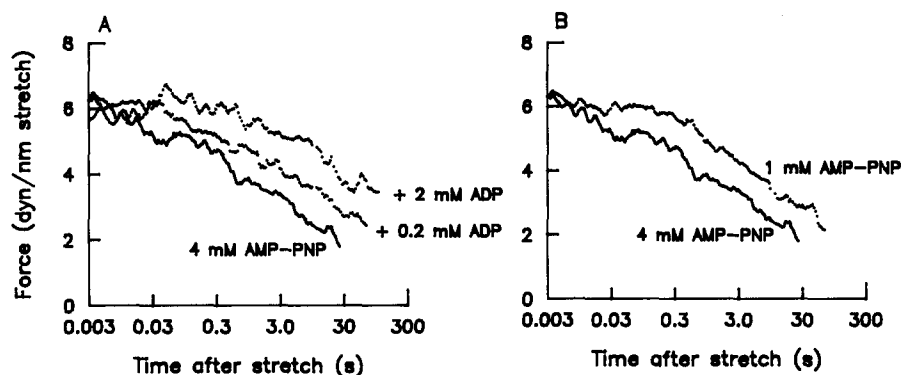


FIGURE 4. Similar to Fig. 3, except that AMP-PNP was used in place of PP_i . Note that all relaxation times are ~ 15 times slower with AMP-PNP than with PP_i , but that the addition of $\sim 0.2 \text{ mM}$ MgADP (A) still mimics the effect of the fourfold reduction in analogue (B). The K_i calculated from these data was $67 \mu\text{M}$. Experiment 010786. Diameter, $50 \times 82 \mu\text{m}$. $P_0 = 46 \text{ dyn}$.

the binding of both the inhibitor and the substrate is noncooperative and also that the effect being measured is linearly proportional to the fraction of cross-bridge heads with bound substrate. In this case, plots of 1/"measured effect" vs. 1/[substrate] will give straight lines. A plot of the slopes of these lines obtained at different inhibitor concentrations, vs. inhibitor concentration, will in turn yield values for K_s , the apparent dissociation constant for substrate binding, and K_i , the inhibition constant for inhibitor binding. In our case, however, where the "measured effect" is the rate of force decay after stretch (a convenient measure of which is $1/\tau_{1/2}$, where $\tau_{1/2}$ is the half-time for force decay), the effect does not follow a simple Michaelis dependence on the MgPP_i or MgAMP-PNP concentration (Schoenberg and Eisenberg, 1985; Anderson, M. L., and M. Schoenberg, manuscript in preparation). Rather, it shows positive cooperativity, so that a plot of 1/"effect" vs. 1/[ligand] is not a straight line, even in the absence of inhibitor. This suggests that one or both of the assumptions usually made in analyzing competitive inhibition is invalid for our case; that is, (a) possibly the binding of ligand is cooperative, i.e., the fraction of heads with bound analogue is not a simple Michaelis function of ligand concentration, or (b) possibly the rate of force decay is not linearly proportional to the fraction of heads with bound ligand. Here we analyze the data with assumption *b*, which we consider to be the more likely assumption. In the Appendix, we show that even if *a* is true, it would have little effect on our conclusions.

If the binding of substrate and inhibitor is noncooperative, the fraction of cross-bridges having bound substrate in the presence of substrate and inhibitor is simply

$$\frac{[\text{AMS}]}{[\text{AM}_{\text{tot}}]} = \frac{1}{1 + (K_s/[\text{S}])(1 + [\text{I}]/K_i)}, \quad (1)$$

where $[\text{AMS}]/[\text{AM}_{\text{tot}}]$ is the fraction of cross-bridge heads with bound ligand, $[\text{S}]$ is the ligand concentration, $[\text{I}]$ is the inhibitor concentration, K_s is the apparent dissociation constant for ligand binding, and K_i is the inhibition constant. Regardless of how nonlinear or cooperative the relationship between $[\text{AMS}]/[\text{AM}_{\text{tot}}]$ and rate of force decay, the effect of an N -fold reduction in the substrate concentration should be the same as the effect of adding inhibitor at a concentration of $(N - 1) \cdot K_i$. This is because the only way that $[\text{S}]$ and $[\text{I}]$ enter into the expression for the decay rate is through the combination $(K_s/[\text{S}])(1 + [\text{I}]/K_i)$ (see Appendix). So, if, as Fig. 3 shows, adding 100–200 μM ADP produces the same effect as reducing the PP_i concentration from 4 to 1 mM, then the K_i , and presumably the K_d for ADP binding, is between $100/3 = 33$ and $200/3 = 67$ μM . The same is true for Fig. 4. Here 200 μM ADP mimics the effect of reducing AMP-PNP from 4 to 1 mM, which again suggests a K_i of 67 μM .

For each experiment, the precise value of K_i was determined as follows. Using two substrate concentrations, first, the half-time for tension decay at the lower substrate concentration was measured in the absence of ADP (see Methods). Then varying amounts of ADP were added to the higher substrate concentration and several half-times were obtained using values of ADP concentration such that the range of half-times obtained included the value obtained at the lower

TABLE II
Calculation of K_i

Analogue	Range	[Mimicking ADP]	Derived K_i
	mM	μ M	μ M
PP _i	4-1	141±33 (4)*	47±11
PP _i	1-0.25	150±23 (3)	50±7.6
PP _i	4-0.25	900±60 (3)	60±4
AMP-PNP	4-1	180±16.5 (5)	60±5.5
			Average 55±4 (15)

*Means ± SEM. Numbers are given in parentheses.

substrate concentration. Finally, the precise concentration of added ADP, giving the same half-time as the lower concentration of substrate, was estimated either by interpolation or occasionally by a small extrapolation. The K_i value was then determined from this concentration by dividing by $(N - 1)$, where N is the ratio of the higher to the lower substrate concentration used.

Table II gives a summary of the results from a number of experiments like those shown in Figs. 3 and 4, where the substrate concentration was varied 4-fold from 4 to 1 mM, and also from a number of experiments where the substrate concentration was varied 4-fold from 1 to 0.25 mM or 16-fold from 4 to 0.25 mM.

If the competition between ADP and PP_i or AMP-PNP is truly competitive, then one should calculate the same value of K_i over each of these concentration ranges. Typical experiments for concentration ranges different from those in Figs. 3 and 4 are shown in Figs. 5 and 6. Fig. 5 shows that adding ADP at a concentration of 100-250 μ M not only mimics the effect of reducing PP_i fourfold over the concentration range from 4 to 1 mM, it mimics the reduction from 1 to 0.25 mM as well. Fig. 6 shows that the effect of reducing the PP_i concentration

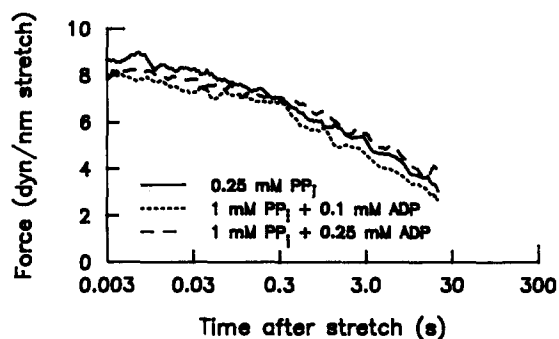


FIGURE 5. Mimicking the effect of a fourfold reduction in MgPP_i from 1 to 0.25 mM by addition of MgADP. The curves show the force relaxation in each of the solutions. Note that adding between 0.1 and 0.25 mM MgADP to 1 mM MgPP_i produced about the same effect as changing PP_i from 1 to 0.25 mM. The K_i calculated for this experiment was 65 μ m. Experiment 121285. Diameter, 83 × 97 μ m. $P_0 = 65$ dyn.

over a 16-fold range from 4 to 0.25 mM is mimicked by the addition of ~1 mM ADP. These results are all compatible with a K_i for ADP inhibition of ~50–100 μM , and, as Table II shows, all the protocols give about the same value for K_i , which supports the idea that the binding of ADP, PP_i , and AMP-PNP is competitive. The average value of K_i from all protocols was $55 \pm 14 \mu\text{M}$.

As stated earlier, the analysis above is based on the assumption that the binding of ADP is noncooperative. However, as shown in the Appendix, if the binding of ADP were cooperative, this would produce very little change in the estimated value of K_i .

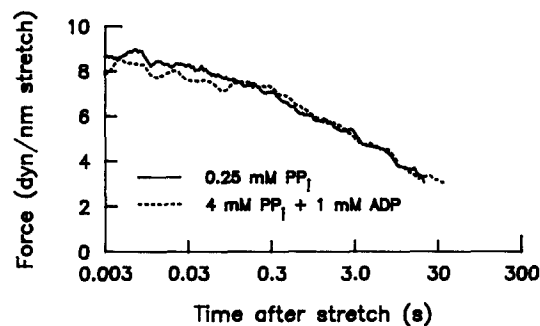


FIGURE 6. Mimicking the effect of a 16-fold reduction in MgPP_i from 4 to 0.25 mM. Note that adding 1 mM ADP to 4 mM PP_i produced about the same rate of force relaxation as decreasing the analogue concentration to 0.25 mM. The K_i calculated from these data is 67 μM . Same fiber as in Fig. 5.

DISCUSSION

Cross-Bridge Detachment Rate Constants in Fibers and in Solution

A major question in muscle contraction is how the rate constants and binding of actin and nucleotides in solution relate to the cross-bridge interactions in fibers. Recently (Schoenberg and Eisenberg, 1985), we showed that detachment of the cross-bridge head in the fiber is complicated compared with that of S1 in solution in that, in the fiber, at least in the presence of ATP analogues, the cross-bridge heads detach not with a single rate constant but with a wide range of detachment rate constants. For a given condition, the fastest rate constants in the fiber are comparable to the detachment rate constant of S1 from actin in solution, but most of the rate constants in the fiber are considerably slower. As Fig. 2 shows, the detachment rate constants with ADP also appear to be slower than in solution. In solution, the detachment rate constants in the presence of ADP are on the order of 0.1 s^{-1} (Marston, 1982; Geeves and Gutfreund, 1982) and fitting the data in Fig. 2 reveals that, in the presence of ADP, less than one-quarter of the cross-bridges can be detaching with rate constants that fast. Unfortunately, the reason for the slowness in the fiber detachment rate constants relative to those of S1 from actin in solution, which is also seen in the absence of nucleotide or analogue, is unknown (Kuhn, 1978; Tozeren and Schoenberg, 1986). Although

it is possible that S1 may be modified during its isolation, it seems unlikely to us that this would account for all the difference between fiber and solution, particularly in light of the similarity between fiber and solution in other regards (see Hibberd and Trentham, 1986).

Our finding that the apparent detachment rate constants in the presence of ADP are not significantly faster than in rigor was unexpected, although not completely surprising. In solution, ADP increases the rate of S1 dissociation from actin only ~10-fold (Marston, 1982). In contrast, AMP-PNP and PP_i , which do cause big increases in the apparent cross-bridge detachment rate constants, cause closer to a 1,000-fold increase in the S1 dissociation rate constant (Marston, 1982; Konrad and Goody, 1982). It is possible that we might have seen a slightly increased rate of force decay in MgADP relative to that in rigor if we had observed the decay over a longer period of time. However, since we do not understand why the rate constants for force decay are so slow, we cannot predict this with certainty.

Nucleotide and Analogue Binding in Fibers and in Solution

The cross-bridge head is a fairly large molecule, and it is conceivable that its energies and motions, and therefore its binding characteristics, might be affected by being constrained in the myofilament lattice. However, compounds like ATP, ADP, and the ATP analogues are small, freely diffusible molecules, and a priori it seems reasonable that the binding of these molecules to an unattached cross-bridge would be similar to their binding to S1 in solution. It is also possible that their binding to an attached cross-bridge might be similar to their binding to acto-S1 in solution.

Initially, it was thought that the binding of AMP-PNP to acto-S1 in solution and to attached cross-bridges in fibers was moderately strong, with dissociation constants in the 100- μ M range (Marston et al., 1976; Konrad and Goody, 1982). Over the years, however, there have been several reports that changing the AMP-PNP concentration in the millimolar range has an effect on muscle fibers (Lymn, 1975; Kuhn, 1978). Indeed, the weight of recent evidence now suggests that AMP-PNP binding in fibers is weak, with a K_d of ~1–5 mM (Pate and Cooke, 1985; Schoenberg and Eisenberg, 1985). Recent evidence also suggests that the binding of AMP-PNP to acto-S1 and myofibrils in solution is weak, again with reported K_d values lying in the 1–5-mM range (Johnson, 1986; Biosca et al., 1986; Sleep and Glyn, 1986). It is not clear whether the wide disparity of results with AMP-PNP has been due to the difficulty in measuring moderately weak binding or whether it is due partly to impurities in commercially available AMP-PNP (Penningroth et al., 1980; Johnson, 1986). However, we feel that the recent evidence suggests that AMP-PNP and PP_i bind with about the same strength in fibers and solution, and that this binding is weak.

The data on ADP binding seem to have been more consistent in showing tight binding in fibers (Marston, 1973; Cooke and Pate, 1985) and in solution (Highsmith, 1976; Greene and Eisenberg, 1980). Nearly all reported K_d values have fallen within a factor of 2 of 100 μ M. Our finding that ADP inhibits AMP-PNP and PP_i with a competitive inhibition constant of 55 μ M agrees with this. It

also suggests that our previously reported effects of AMP-PNP and PP_i are due to binding at the active site and are not due to nonspecific charge effects. If the effects of AMP-PNP and PP_i were due to nonspecific charge effects, a small amount of additional ADP would not inhibit them.

Cooperativity in the Effect of Analogue Binding

Recently (Schoenberg and Eisenberg, 1985; Anderson, M. L., and M. Schoenberg, manuscript in preparation), we have shown that the analogue concentration dependence of the rate of force decay after stretch shows positive cooperativity. One possible explanation for this is that the cooperativity is in the binding of the analogue; that is, the fraction of cross-bridge heads with bound analogue is not a simple Michaelis function of the analogue concentration. Another possibility is that the cooperativity is in the effect of analogue binding; this would be the case, for example, if the rate of force decay were not directly proportional to the fraction of cross-bridge heads with bound analogue. Since, in solution, there appears to be little evidence for cooperative binding of analogues, we favor an explanation of the latter type.

One way of explaining the cooperativity in the analogue concentration dependence of the rate of force decay without involving cooperative analogue binding is to assume that a cross-bridge cannot relax the force it supports unless both S1 heads of the cross-bridge have bound analogue (Anderson, M. L., and M. Schoenberg, manuscript in preparation). Since previously (Schoenberg, 1985) we showed that the decay of force could be understood in terms of cross-bridge heads detaching and then reattaching in positions of lesser strain, this model for explaining the cooperativity can be thought of as one in which a single cross-bridge head cannot detach and relocate itself in a position of lesser strain unless the other head of the myosin molecule also binds analogue and the two detach simultaneously. In this instance, the rate of force decay would be proportional, not to the fraction of heads having bound analogue, but to the fraction of cross-bridges having two analogue molecules bound. In this case, the concentration dependence of the rate of force decay would show cooperativity, with a Hill coefficient of 2, in agreement with the data (Anderson, M. L., and M. Schoenberg, manuscript in preparation).

What is interesting about this cooperative model is that it might also conceivably explain why ADP does not accelerate the rate of force relaxation on a time scale of several minutes. Although the detachment rate constant of S1·ADP from actin in solution is on the order of 0.1 s^{-1} (Marston, 1982; Geeves and Gutfreund, 1982), if both heads of the cross-bridge needed to bind analogue and detach simultaneously before force could be relieved, this process would presumably be much slower than 0.1 s^{-1} . This might especially be true under conditions such as ours, where cross-bridge binding may be not very much less than in rigor (Biosca et al., 1986).

In the Appendix, we consider the less likely case in which the cooperativity under discussion is not in the effect of binding of ligand but is in the actual binding itself. One conclusion reached is that even if the binding of ligand were cooperative, the calculated value of the K_i for ADP inhibition would still be

similar in magnitude to the value derived in Table II. A second conclusion is that if the binding of AMP-PNP or PP_i were cooperative, the binding of ADP would also have to be cooperative. This follows from the fact that if PP_i or AMP-PNP binding were cooperative but ADP binding were not, the amount of ADP that would have to be added to 4 mM analogue to mimic the effect of an N -fold reduction in analogue would not be proportional to N as observed. Instead, it would be proportional to some power of N .

In summary, all our results are compatible with the idea that ADP, AMP-PNP, and PP_i bind competitively and noncooperatively to the active site of attached cross-bridges, doing so with K_d values similar to those found for their binding to acto-S1 in solution (50–100 μ M for ADP; 1–5 mM for AMP-PNP and PP_i). In contrast to the behavior of AMP-PNP and PP_i , the binding of ADP to the active site does not seem to greatly increase the cross-bridge detachment rate constants relative to those in rigor.

APPENDIX

Here the standard equations for competitive inhibition are derived without the usual assumption that the binding of ligand and inhibitor is noncooperative. Cooperativity is often expressed in terms of the Hill equation where, if the binding, $E + S \rightleftharpoons ES$, is cooperative, then θ , the fractional saturation of E with substrate, can be approximated according to the relationship

$$\theta = \frac{[ES]}{[ES] + [E]} = \frac{([S]/K)^n}{1 + ([S]/K)^n}, \quad (\text{A1})$$

where [S] is the concentration of substrate, K is a (dissociation) constant, and n is an interaction parameter defining the degree of cooperativity.

Inverting both sides of Eq. A1 and subtracting 1 from each side yields the simpler relationship

$$[E]/[ES] = (K/[S])^n.$$

For our particular case, substrate and inhibitor binding to the actomyosin cross-bridge, we can write

$$\frac{[AM]}{[AMS]} = \left(\frac{K_s}{[S]}\right)^{n_s} \quad (\text{A2})$$

$$\frac{[AM]}{[AMI]} = \left(\frac{K_i}{[I]}\right)^{n_i}, \quad (\text{A3})$$

where [AM] is the concentration of cross-bridge heads free of substrate or inhibitor, [S] and [I] are, respectively, the concentrations of substrate and inhibitor, [AMS] and [AMI] are, respectively, the concentration of heads with bound substrate and inhibitor, K_s and K_i are, respectively, the dissociation constants for substrate and inhibitor binding, and n_s and n_i are, respectively, the interaction parameters for the cooperative binding of substrate and inhibitor (Monod et al., 1965).

If all the cross-bridge heads are assumed to be bound to actin (White, 1970; Schoenberg and Eisenberg, 1985; Brenner et al., 1986*b*),

$$[AM] + [AMS] + [AMI] = [AM_{\text{tot}}], \quad (\text{A4})$$

where $[AM_{\text{tot}}]$ is the total concentration of cross-bridge heads.

Substituting Eqs. A2 and A3 into Eq. A4 and rearranging yields

$$\frac{[\text{AMS}]}{[\text{AM}_{\text{tot}}]} = \frac{1}{1 + (K_s/[\text{S}])^{n_s} \{1 + ([\text{I}]/K_i)^{n_i}\}} \quad (\text{A5})$$

Note that when $n_s = n_i = 1$, meaning that the binding of substrate and inhibitor is not cooperative, Eq. A5 reduces to the standard equation for competitive inhibition (Eq. 1 of the text). The first important thing to note about Eq. A5 is that as long as the rate of force decay is a function, whether linear or nonlinear, of $[\text{AMS}]/[\text{AM}_{\text{tot}}]$, then an N -fold reduction in substrate will be mimicked by the addition of inhibitor at a concentration of

$$K_i \cdot (N^{n_s} - 1)^{1/n_i}.$$

n_s and n_i are a measure of the extent of cooperativity in the binding of ligand, larger values implying greater cooperativity. When $n_s = n_i = 2$, a fourfold reduction in substrate will be mimicked by a $3.87 \cdot K_i$ addition of inhibitor. This compares with a $3 \cdot K_i$ addition of inhibitor for noncooperative binding. If $n_s = n_i = 3$, a fourfold reduction in substrate is mimicked by a $3.98 \cdot K_i$ addition of inhibitor and, in fact, in the limit as $n_s = n_i \rightarrow \infty$, a fourfold reduction in substrate is mimicked by a $4 \cdot K_i$ addition of inhibitor. This means that even if the binding of substrate and inhibitor were extremely cooperative, our estimate of K_i based on the assumption of noncooperative binding would be in error by only 33%.

A second point to note is that if the binding of substrate were cooperative, $n_s = 2$, but that for inhibitor were noncooperative ($n_i = 1$), a 4-fold reduction in substrate would be mimicked by a $15 \cdot K_i$ addition of inhibitor and a 16-fold reduction in substrate would be mimicked by a $255 \cdot K_i$ addition of inhibitor. Our experimental finding that the amount of ADP that must be added to a mimic a reduction of a factor of 4 or factor of 16 in substrate is more or less proportional to the reduction factor rules out this possibility.

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REFERENCES

- Biosca, J. A., L. E. Greene, and E. Eisenberg. 1986. Binding of ADP, AMP-PNP and PP_i to crosslinked and non-crosslinked Acto S-1. *Journal of Biological Chemistry*. 261:9793-9800.
- Brenner, B., J. M. Chalovich, L. E. Greene, E. Eisenberg, and M. Schoenberg. 1986a. Stiffness of skinned rabbit psoas fibers in MgATP and MgPP_i solution. *Biophysical Journal*. 50:685-691.
- Brenner, B., L. C. Yu, L. E. Greene, E. Eisenberg, and M. Schoenberg. 1986b. Ca²⁺-sensitive cross-bridge dissociation in the presence of magnesium pyrophosphate in skinned rabbit psoas fibers. *Biophysical Journal*. 50:1101-1108.
- Cooke, R., and E. Pate. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophysical Journal*. 48:789-798.
- Eastwood, A. B., D. S. Wood, K. L. Bock, and M. M. Sorenson. 1979. Chemically skinned mammalian skeletal muscle. 1. The structure of skinned rabbit psoas. *Tissue and Cell*. 11:533-556.
- Geeves, M. A., and H. Gutfreund. 1982. The use of pressure perturbations to investigate the interaction of rabbit muscle myosin subfragment 1 with actin in the presence of MgADP. *FEBS Letters*. 140:11-15.
- Goody, R. S., J. Barrington Leigh, H. G. Mannherz, R. T. Tregear, and G. Rosenbaum. 1976. X-ray titration of binding of β , γ -imido-ATP to myosin in insect flight muscle. *Nature*. 262:613-615.

- Greene, L. E., and E. Eisenberg. 1980. Dissociation of the actin-subfragment 1 complex by adenylyl-5'-yl imidodiphosphate, ADP, and PP_i. *Journal of Biological Chemistry*. 225:543-548.
- Hibberd, M. G., and D. R. Trentham. 1986. Relationships between mechanical and chemical events during muscular contraction. *Annual Review of Biophysical Chemistry*. 15:119-161.
- Highsmith, S. 1976. Interactions of the actin and nucleotide binding sites on myosin subfragment one. *Journal of Biological Chemistry*. 251:6170-6172.
- Hofmann, W., and R. S. Goody. 1978. Ternary complex formed between actin, myosin subfragment 1 and ATP (β , γ -NH). *FEBS Letters*. 89:169-173.
- Johnson, R. E. 1986. The effect of ethylene glycol and Ca²⁺ on the binding of Mg²⁺ adenylyl-5'-yl imidodiphosphate to rabbit skeletal myofibrils. *Journal of Biological Chemistry*. 261:728-732.
- Johnson, R. E., and P. H. Adams. 1984. ADP binds similarly to rigor muscle myofibrils and to actomyosin-subfragment one. *FEBS Letters*. 174:11-14.
- Kawai, M. 1986. The role of orthophosphate in crossbridge kinetics in chemically skinned rabbit psoas fibres as detected with sinusoidal and step length alterations. *Journal of Muscle Research and Cell Motility*. 7:421-434.
- Konrad, M., and R. S. Goody. 1982. Kinetic and thermodynamic properties of the ternary complex between F-actin, myosin subfragment 1 and adenosine 5'-[β , γ -imido]triphosphate. *European Journal of Biochemistry*. 128:547-555.
- Kuhn, H. J. 1978. Cross-bridge slippage induced by the ATP analogue AMP-PNP and stretch in glycerol-extracted fibrillar muscle fibres. *Biophysics of Structure and Mechanism*. 4:159-168.
- Lymn, R. W. 1975. Low-angle X-ray diagrams from skeletal muscle: the effect of AMP-PNP, a non-hydrolyzed analogue of ATP. *Journal of Molecular Biology*. 99:567-582.
- Lymn, R. W., and E. W. Taylor. 1971. Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry*. 10:4617-4624.
- Marston, S. 1973. The nucleotide complexes of myosin in glycerol-extracted muscle fibres. *Biochimica et Biophysica Acta*. 305:397-412.
- Marston, S. B. 1982. The rates of formation and dissociation of actin-myosin complexes. *Biochemical Journal*. 203:453-460.
- Marston, S. B., C. D. Rodger, and R. T. Tregear. 1976. Changes in muscle crossbridges when β , γ -imido-ATP binds to myosin. *Journal of Molecular Biology*. 104:263-276.
- Millar, N. C., and M. A. Geeves. 1983. The limiting rate of the ATP-mediated dissociation of actin from rabbit skeletal muscle myosin subfragment 1. *FEBS Letters*. 160:141-148.
- Monod, J., J. Wyman, and J. Changeux. 1965. On the nature of allosteric transitions: a plausible model. *Journal of Molecular Biology*. 12:88-118.
- Mornet, D., R. Bertrand, P. Pantel, E. Audemard, and R. Kassab. 1981. Structure of the actin-myosin interface. *Nature*. 292:301-306.
- Pate, E., and R. Cooke. 1985. The inhibition of muscle contraction by adenosine 5' (β , γ -imido)triphosphate and by pyrophosphate. *Biophysical Journal*. 47:773-780.
- Penningroth, S. M., K. Olehnik, and A. Cheung. 1980. ATP formation from adenylyl-5'-yl imidodiphosphate, a nonhydrolyzable ATP analogue. *Journal of Biological Chemistry*. 255:9545-9548.
- Persecchini, A., J. T. Stull, and R. Cooke. 1985. The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers. *Journal of Biological Chemistry*. 260:7951-7954.
- Schoenberg, M. 1985. Equilibrium muscle crossbridge behavior: theoretical considerations. *Biophysical Journal*. 48:467-475.

- Schoenberg, M. 1987. The kinetics of weakly- and strongly-binding crossbridges: implications for contraction and relaxation. *In* The Molecular Mechanism of Muscle Contraction. H. Sugi and G. H. Pollack, editors. Plenum Publishing Corp., New York. In press.
- Schoenberg, M., and E. Eisenberg. 1985. Muscle cross-bridge kinetics in rigor and in the presence of ATP analogues. *Biophysical Journal*. 48:863-871.
- Sleep, J., and H. Glyn. 1986. Inhibition of myofibrillar and actomyosin subfragment 1 adenosinetriphosphatase by adenosine 5'-diphosphate, pyrophosphate and adenylyl-imidodiphosphate. *Biochemistry*. 25:1149-1154.
- Tozeren, A., and M. Schoenberg. 1986. The effect of crossbridge clustering and head-head competition on the mechanical response of skeletal muscle under equilibrium conditions. *Biophysical Journal*. 50:875-884.
- White, D. C. S. 1970. Rigor contraction and the effect of various phosphate compounds on glycerinated insect flight and vertebrate muscle. *Journal of Physiology*. 208:583-605.