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THE EFFECT OF AN ADP ANALOG ON ISOMETRIC FORCE AND ATPase ACTIVITY OF ACTIVE MUSCLE FIBERS

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Running Head:

Perturbation of muscle contraction by an ADP analog

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ABSTRACT

The role played by ADP in modulating cross-bridge function has been difficult to study, as it is hard to buffer ADP concentration in skinned muscle preparations. To solve this, we used an analog of ADP, spin-labeled ADP (SL-ADP). SL-ADP binds tightly to myosin but is a very poor substrate for creatine kinase or pyruvate kinase. Thus, ATP can be regenerated allowing well-defined concentrations of both ATP and SL-ADP. We measured isometric ATPase rate and isometric tension as a function of both [SL-ADP], 0.1-2 mM, and [ATP], 0.05-0.5 mM, in skinned rabbit psoas muscle, simulating fresh or fatigued states. Saturating levels of SL-ADP increased isometric tension (by P'), the absolute value of P' being nearly constant, ~ 0.04 $N.mm^{-2}$, in variable ATP levels, pH 7. Tension decreased (50 - 60%) at pH 6, but upon addition of SL-ADP P' was still ~ 0.04 $N.mm^{-2}$. The ATPase was inhibited competitively by SL-ADP with an inhibition constant, K_i, of approx. 240 and 280 μM at pH 7 and 6 respectively. Isometric force and ATPase activity could both be fit by a simple model of cross-bridge kinetics.

INTRODUCTION

During skeletal muscle fatigue, several major mechanical and energetic changes take place. These include a decrease in the maximal tension (P_0), a slower maximal contraction velocity (V_{max}), a slower rate of relaxation (15, 16, 24) and a higher tension economy, i.e. more tension generated per ATP hydrolyzed (9, 10). A popular hypothesis is that many of these changes may be due to the effects of increased levels of specific metabolites, including ADP, on muscle cross-bridges. However, data to support this hypothesis have not been unambiguous, and many aspects of fatigue remain unexplained.

Studies of fatigue *in vivo*, are complex, because numerous metabolites change, making the correlation with the alterations of the properties of the muscle difficult to establish (for review see 12, 20). To delineate the effects of a single metabolite, such as ADP, permeabilized isolated fibers have been studied *in vitro* (8, 13, 19, 23, 27, 30). Measurements made at 10-15 ºC suggested that the drop in tension is probably due to the lower pH and higher free inorganic phosphate concentration ([P_i]) observed during fatigue (5, 28, 31, 42), but these effects are much reduced at more physiological temperatures (34, 42, 44, 41). A lower pH is also known to decrease contraction velocity however, it does not completely account for the drop in V_{max} seen during fatigue (34, 42).

According to the accepted model of the acto-myosin cross-bridge cycle, ADP, which increases from 20 to 200 µM during fatigue (14), should compete with ATP for the ATP binding site on myosin and thereby slow down cross-bridge detachment (13, 36). This in turn should inhibit ATPase activity and contraction velocity and should enhance

isometric tension. Together these effects would increase the economy of contraction. However, to date the observed effects of increased ADP on the tension and ATPase activity, although in the right direction, have been found to be too small to make a significant contribution (4, 8, 13, 29, 30, 40).

In skinned muscle studies, an ATP regenerating system (with creatine kinase, CK or pyruvate kinase, PK) can be used to maintain ATP concentrations within the fiber. However if one were to add additional ADP to a solution with an ATP regenerating system, it would quickly be phosphorylated to ATP leaving little time to observe the effects of increasing ADP on fiber mechanics. To circumvent this problem, investigators have used high concentrations of nucleotides in the absence of a regeneration system, and estimated intracellular concentrations by analyzing the diffusion of nucleotides (8). Photolysis of caged compounds has also been used for instantaneous generation of ADP (29). Or, higher ADP concentrations have been generated by addition of extra creatine (Cr) to the bathing solution (4).

We have recently come up with one solution which facilitates the study of the effects of ADP in skinned muscle preparations, while controlling other nucleotide concentrations with a regeneration system. We have found that attachment of a spin label, to the 2' or 3' positions on the ribose of ATP, greatly inhibits interaction with CK or PK. This analog binds to myosin and to acto-myosin with an affinity that is equal to that of ADP (11), and mechanical data presented here reconfirmed this conclusion. Thus we were able to use an ATP regenerating system to maintain a well-defined concentration of ATP within the fiber, avoiding the buildup of ADP from hydrolysis of ATP, and we could then measure the mechanical responses of the fiber over wide

ranges of [ATP] and [SL-ADP]. We determined the effects of SL-ADP on ATPase activity and tension at varying concentrations of ATP, finding that SL-ADP changes ATPase activity and tension in a competitive manner. The cross-bridge interactions are also affected by lowering of pH and/or accumulation of P_i, discussed above, and under such conditions, we hypothesized that the effects of SL-ADP may be different than those obtained at control conditions (i.e. high ATP, low, P_i, pH 7).

The results can be explained by a simple model in which SL-ADP competes with ATP at the end of the power stroke. An adequate fit to the data required that cross-bridges in force generating states were capable of rapid exchange with pre-power stroke cross-bridges. Thus the transition from pre-power stroke cross-bridges to force generating cross-bridges can be reversed by binding of SL-ADP, which occurs at the end of the power stroke.

METHODS

Fiber Mechanics. Rabbits (New Zealand White) were heavily sedated and euthanized according to the guidelines of the Institutional Animal Care and Use Committee for the University of California, San Francisco. Psoas fibers were harvested and chemically skinned as described previously (7). For mechanical experiments, single fibers were dissected from a bundle of fibers on a cold stage whilst still immersed in the skinning solution. A single fiber was then mounted between a solid-state force transducer and a rapid motor for changing fiber length, as previously described (7), using Duco Cement (Dencon Consumer Products, Danvers, MA 01923, USA) diluted in acetone. The apparatus had two temperature-controlled wells and fibers could be rapidly switched between solutions. The fiber was then lowered into a relaxing solution, and fiber length and thickness were measured. The length of the unfixed portion of the fiber between the arms varied from 3 to 6 mm, fiber diameter ranged from 50 to 80 μm, and initial sarcomere length ranged from 2.2-2.4 μm.

A common protocol was to first immerse the fiber in a relaxing solution (well 1) and allow complete perfusion of CK and phosphocreatine (PCr) or PK and phosphoenol pyruvate (PEP) and nucleotides (approximately 3 min). The fiber was then switched to an activating solution in well 2, and mechanical measurements were made. Following measurements, the fiber was returned to well 1 and the solution in well 2 was replaced by the same fresh or another appropriate experimental buffer. Mechanical measurements were repeated and the fiber returned to the relaxing solution. The solution in well 2 was changed again and mechanical measurements were repeated for a last time under control conditions to determine stability of the fiber. A 10% decline in

P_0 was set as our performance criterion. Damaged fibers were discarded. All fiber experiments were done at 10 °C in both wells and the bathing solutions were continuously stirred.

Solutions. The basic rigor buffers contained in mM: 120 potassium acetate (KAc), 5 $MgCl_2$, 1 EGTA, and either 50 MOPS, pH 7 or 50 MES, pH 6. Phosphate (3-54 mM) was added to this solution, keeping the ionic strength constant by changing KAc (35). A relaxing solution was achieved by addition of 0.05-0.5 mM ATP and either 20 mM PCr with 1 $mg.ml^{-1}$ CK or 20 mM PEP with 1 $mg.ml^{-1}$ PK. These solutions were made fresh on each experimental day. Activating solution was achieved at pCa 4.5. A range of SL-ADP concentrations was used (0.1 to 2 mM) to examine the effects of the ADP analog on mechanical fiber properties. The concentration of free Mg^{++} varied in our experiments from 3.8 to 4.9 mM. Variation of Mg^{++} in this range had no perceptible effect on fiber function. For some experiments, involving measurements of ATPase activity at very low [ATP], trace contamination of ADP in the SL-ADP, ~0.1-0.5 %, was removed by treatment of the stock solution of SL-ADP with 1 $mg.ml^{-1}$ myofibrils and 0.02 $mg.ml^{-1}$ adenylate kinase for 10 minutes at room temperature. This incubation hydrolyzes ADP to AMP, but does not affect SL-ADP. The activity of the adenylate kinase was then inhibited by addition of 100 μM diadenosine pentaphosphate, the myofibrils were removed by centrifugation, and the purified SL-ADP was used. All reagents were purchased from Sigma (St Louis, MO, USA).

Enzymatic studies. In addition to mechanical experiments, the ATPase activities of rabbit psoas myofibrils (prepared basically as described by Etlinger et al (18) were measured by direct determination of NADH depletion linked to ADP production,

described in more detail previously (33). Activating solutions contained 10 mM PEP, 0.25 mM β-NADH and in $mg.ml^{-1}$, 0.07 lactate dehydrogenase (LDH) and 0.2 pyruvate kinase. The activities of the enzymes used were in $\mu moles.min^{-1}.mg^{-1}$ protein: CK, 200 (pH 7, 37 ^{o}C); PK, 430 (pH 7.6, 37 ^{o}C); LDH, 940 (pH 7.5, 37 ^{o}C).

Spin labeled ADP (SL-ADP). SL-ADP was synthesized according to Crowder and Cooke (11). Both thin layer chromatography (TLC) and mechanical measurements revealed that SL-ADP was very slowly phosphorylated to SL-ATP by CK. Enzyme activity was tested by incubating the SL-ADP with the enzyme in rigor buffer, at 10 ^{o}C. At specified times an alliquot was removed and spotted on a pre-coated high performance TLC aluminum plate (silica gel, 60 F254, 0.2 nm, Merck KGaA, Darmstadt, Germany) along with appropriate controls. The plate was well dried and developed in an isopropanol: NH_4OH:H_2O solution (6:3:1 v/v) at room temperature. After development the spots corresponding to SL-ADP and SL-ATP were scraped and extracted in 600 mM KCl, 50 mM MOPS, pH 7.5, and the presence and quantity of SL-nucleotide was measured by EPR spectroscopy (ER/200D IBM Instruments, Inc. Danbury, CT). A 1 mM solution of SL-ADP was approximately half-phosphorylated, 50 ± 12% (n=4), in 15 minutes at 10 ^{o}C by 1 $mg.ml^{-1}$ CK. This rate is only 0.06% of the activity of CK for ADP under similar conditions. For our mechanical experiments performed at 10 ^{o}C within 90 seconds of adding SL-ADP, the conversion rate was slow enough that negligible SL-ATP would be generated within the fiber. In addition, during the course of the experiments we discovered that SL-ADP was not phosphorylated by the PK-PEP system, within the accuracy of our measurements (again TLC, EPR and mechanical

measurements). Thus we switched to the PK-PEP system, which gave identical results as the CK-PCr system, but allowed SL-ADP solutions to be used for a longer time.

ATP regeneration system. To verify the capability of 1 $mg.ml^{-1}$ PK to maintain ATP concentration and eliminate ADP we measured force (n=6) at three conditions, in the presence of 150 μM ATP, 3 mM P_i and 20 mM PEP, pH 7. We compared force generated with 1 $mg.ml^{-1}$ PK vs. 2 $mg.ml^{-1}$ PK, which gave forces of 0.093 ± 0.003 and 0.092 ±0.002 $N.mm^{-2}$ respectively; we also compared force generated with 1 $mg.ml^{-1}$ PK, and then with addition of 1mM ADP, which resulted in forces of 0.096 ± 0.003 and 0.094 ± 0.005 $N.mm^{-2}$ respectively. In the latter comparison, a small drop in force was observed which was expected as the PK rapidly rephosphorylated the added ADP to ATP, thus making more ATP available to the fiber. We estimated internal [ADP] to be less than 25 μM based on a calculation of the production, consumption and diffusion of ADP following the equations of reference (8). Thus in the experiments described here, competition from internally generated ADP is much less than from added SL-ADP.

Data reduction. Force data for each condition, in $N.mm^{-2}$, were averaged and expressed as mean (± SD), n = number of fibers used, when reported. In the case of parameters based on fits, the ± 95% confidence interval is given (i.e. when reporting inhibition or dissociation constants). Mean force data were fit assuming that the effect of SL-ADP could be expressed as a simple binding isotherm:

$$P_{SL\text{-}ADP} = P_0 + P' \bullet [SL\text{-}ADP] / (K_d^{app} + [SL\text{-}ADP]) \qquad 1.$$

Where $P_{SL\text{-}ADP}$ is the tension as a function of [SL-ADP], P_0 is the maximum isometric tension in the absence of SL-ADP, P' is the increment in tension approached as SL-

ADP increases to saturating levels and K_d^{app} is an apparent dissociation constant that describes the strength of the binding of SL-ADP in competition with ATP.

Model. Simple 3-and 4-state models of cross-bridge kinetics were analyzed using commercially available software (Berkeley Madonna, 8.0.1. © 1996- 2000 R.I. Macey & G.F. Oster). The kinetic rates were determined from the experimental data obtained for force and ATPase activity in our isometric fibers and myofibrils. For a 4-state model, cross-bridges are assumed to be in one of 4 states: (1) detached from actin, or attached weakly without generating force, (2) attached to actin in the power stroke, (3) attached to actin in the state achieved following binding of SL-ADP at the myosin nucleotide site, (4) attached to actin with no nucleotide at the myosin site. States 2, 3, 4 all generate force. We envision the following cross-bridge cycle. Cross-bridges start in state 1 having ATP or ADP. P_i at the nucleotide site and do not generate force. The transition to state 2 involves some conformational change in myosin, associated with the release of P_i that produces actin-attached force generating cross-bridges. State 2 is in reality a mixture of different states, and ADP release and binding involves one of these, which is state 3 in the model. Release of ADP from state 3 leads to the rigor state, 4. The binding of ATP to state 4 returns the cross-bridge to state 1. The force generated by the model is a function of the populations in the force generating states, states 2, 3, and 4, and the amount of force generated by a cross-bridge in each one of these states. The magnitudes of the relative forces generated by cross-bridges in states 2, 3 and 4 are not known. We assume here that the transitions between states 2, 3 and 4 do not change the conformation of the cross-bridge so that all these states generate the same force. Release of ADP from force generating cross-bridges does not change the strain

on the cross-bridge, so that rigor states (state 4) maintain the same tension as states 2 or 3. However the effect of relaxing this assumption did not change the conclusions. At high levels of ATP the population of states 3 and 4 are small, and the fraction of force generating cross-bridges is given by the steady state distributions of states 1 and 2. For a 3-state model states 2 and 3 were combined. As we will discuss later, a 4-state model was more applicable (Fig. 5). ATPase activity was calculated as the flux from state 4 back to state 1. The model was not sensitive to the exact values of the rate constants connecting states 1, 2 and 3, as long as certain conditions were satisfied. In particular the reverse transitions had to be more rapid than the flux through the states, or P' would not be a constant. The values of k_{12}, k_{21}, k_{23}, k_{32} were set so that the ATPase activity was 1 s^{-1} and the fraction of the non-force state, state 1 was 50% at high [ATP]. The assumption that 50% of the cross-bridges generate force is arbitrary, but the exact value is not crucial, and the data could be fit equally well assuming other values, e.g. 25%. In the simulations shown all four rate-constants were set to 3 s^{-1}. However the conclusions were similar if they were varied between 1 and 10, keeping the flux through them at the assumed ATPase rate of 1 s^{-1}, using variable ratios of forward to backward rate constants to keep the flux constant.

The rate constant involving the binding of ATP was set by a fit to the ATPase activity. The rate constant k_{41} involves the binding of ATP, and thus it depends directly on the concentration of ATP. The magnitude of this rate constant can be determined from the dependence of the ATPase activity on [ATP]. When the concentration of ATP is equal to K_m the rate k_{41} must be such that the average time required to make the transition from state 4 to state 1, 1/ k_{41}, must be equal to the cycle time at saturating

ATP, 1 second. The observed value of K_m at pH 7 sets the second order binding constant for ATP to be 3.6×10^4 M^{-1} s^{-1}. Thus k_{41} = $3.6 \times 10^4 \times$ [ATP] s^{-1}, where [ATP] is in molar. The rates of binding or release of ADP from the rigor state of active cross-bridges is not known. In the model the rate of release was set at 50 s^{-1}, and the conclusions were not changed if this value was 10 times smaller or larger. The transition involving the binding of SL-ADP, k_{43}, is directly proportional to the concentration of SL-ADP. This rate constant was set using the known affinity of SL-ADP for the rigor state, 5×10^3 M^{-1} (reference (11) and more recent measurements, data not shown). Thus, k_{43} = $2.5 \times 10^5 \times$ [ADP] s^{-1}, where [ADP] is in molar. These rates change when the pH is decreased to 6.

RESULTS

SL-ADP Increases Isometric Tension. In analyzing SL-ADP's effect on isometric tension, we activated a fiber and allowed it to rise to a stable peak force (P_0). We then added SL-ADP and allowed the tension increase to reach a stable value. A sample data set is presented in Figure 1 where we added 0.5 mM of SL-ADP to a fiber activated in 50 µM ATP. The fiber could then be returned to a bath that lacked SL-ADP and tension returned to control values, showing that the increase was reversible.

In Figure 2 we present the effect of adding 0.1 - 2.0 mM SL-ADP to fibers at 50, 150, or 500 µM ATP. At low [ATP] the fiber tension was high and it decreased with increasing [ATP]. Addition of SL-ADP increased tension at all values of ATP, as shown in Figure 2 and Table 1. At lower concentrations of ATP, the same increase in tension required lower concentrations of SL-ADP. This is expected if the increase in tension is

a result of SL-ADP acting as a competitive inhibitor of the binding of ATP and the subsequent dissociation of myosin heads from actin.

Also shown in Figure 2 are fits to the data, assuming that the effect of SL-ADP could be expressed as a simple binding isotherm (see Equation 1 in methods). This simple approach provided a good fit to the data under all conditions providing values for the two free parameters, K_d^{app} and P', shown in Table 1. K_d^{app} is an apparent dissociation constant that describes the strength of the binding of SL-ADP in competition with ATP. The value of K_d^{app} increased as [ATP] increased, as expected for competition between SL-ADP and ATP. The tension increase, P', reached a plateau that was approximately 0.04 $N.mm^{-2}$ greater than the P_0 observed in the absence of SL-ADP, for variable [ATP] and pH (Table 1). Notably, in the presence of added phosphate the value of P' decreased (Table 1, Fig. 3). The near constant value of P' found as [ATP] varied is not expected from simple models of cross-bridge action, which predict that the tension increase should be lower at lower [ATP], where the initial tension is higher due to the presence of rigor cross-bridges.

The relationship between P_0 and P' is not linear. As noted above, increasing ATP lowered P_0 but not P', with the ratio P'/P_0 equal to 0.37, 0.41 and 0.62 for [ATP] of 50, 15 and 500 μM respectively. Increasing phosphate or decreasing pH from 7 to 6, decreased isometric tension production (Table 1), as expected from previous reports. For example, reduction in pH from 7 to 6 (3 mM P_i) produced a 67.5 ± 2.3 % force decline (or 0.060 ± 0.021 $N.mm^{-2}$, n=3). Addition of saturating SL-ADP gave a ratio of 1.37, which is greater than observed at 7. Addition of 54 mM P_i also decreased P_0 and increased the ratio P'/P_0 to 1.0.

The conditions of low pH and high P_i employed in our study were intended to simulate the conditions found in muscle fibers during severe muscle fatigue, and thus are of interest in understanding the role of increased concentrations of SL-ADP (and thus ADP) in this state of the muscle. As can be seen in Table 1 and Figure 2, SL-ADP produces the same absolute increase in tension at the lower pH as is seen at pH 7 (at 3 mM P_i). Thus SL-ADP produces a larger relative increase in tension at the lower pH. In contrast, the addition of SL-ADP to fibers incubated in high phosphate produces a smaller P' than is seen at lower concentrations of phosphate (Table 1, Fig. 3). Although the change in P_0 was approximately the same for pH 6, 3 mM P_i and for pH 7, 54 mM P_i, the value of P' in the later case was less than half that in the former, see Table 1. The smaller value of P' found in the presence of high P_i is also observed at pH 6. At both pH 7 and at 6 the inhibition of P_0 by P_i tended to be greater than the inhibition of P'. These data suggest that the inhibition in P_0 caused by P_i is due to a different mechanism than inhibition in P_0 caused by H^+ accumulation.

Comparison with previous results. In a number of previous investigations the effect of ADP on fiber tension was measured by addition of relatively high, millimolar, concentrations of ADP to fibers activated in millimolar ATP (8, 13, 27, 29, 30, 40). These investigations have found that tension increases by about 10% on addition of 1 mM ADP to fibers activated in 3-5 mM ATP. To determine whether SL-ADP would have the same effect on tension as seen in these earlier studies, we added 1 mM SL-ADP or 1 mM ADP to fully activated fibers in 3 mM ATP and 3 mM P_i, in the absence of an ATP regenerating system (pH 7, 10°C). The average increase in tension was 12 ± 5% for SL-ADP or 10 ± 2.5% for ADP (n = 3). The value we obtain for ADP is similar to the

results of previous investigations, and is similar to that of SL-ADP. The equivalence of the force increase found for SL-ADP and ADP further shows that the presence of the label on the ribose does not alter the binding of the analog to myosin in active fibers, as initially reported by Crowder and Cooke (11) in an EPR spectra analysis. Moreover, mant-ADP, a fluorescent analog that is similar in structure to SL-ADP also binds to myosin similarly to ADP (45).

SL-ADP Decreases Isometric ATPase activity. The effect of SL-ADP on the ATPase activity was determined using myofibrils from psoas muscle. The myofibrils were first cross-linked lightly with gluteraldehyde to prevent shortening, so that they were a reasonable model of an isometric muscle fiber (22). The ATPase activity was determined as a function of concentration for both ATP and SL-ADP. Our data showed that SL-ADP acted as a competitive inhibitor of the binding of the substrate ATP (Fig. 4). Competitive inhibition is described by the equation below:

$$\text{ATPase} = V_{max}.[\text{ATP}]/(K_m(1+[\text{SL-ADP}]/K_i) + [\text{ATP}]) \qquad 2.$$

Where V_{max} is the activity at infinite [ATP], K_m is the Michaelis constant describing the binding of the substrate ATP and K_i is the inhibition constant describing the strength of the binding of the inhibitor SL-ADP. In the presence of a concentration of SL-ADP equal to K_i , the ATPase follows Michaelis-Menten kinetics with an apparent K_m that is twice the K_m in the absence of SL-ADP. In pH 7 (Fig. 4A), our results defined a K_m for the ATPase activity of 29 ± 8 μM and a K_i for the inhibition of the ATPase activity by SL-ADP of 240 ± 20 μM. Similar experiments performed at pH 6 (Fig. 4B) revealed a decrease in the value of K_m to 4.8 ± 2 μM; however the value of K_i remained approximately the same at 280 ± 50 μM, indicating that ATP bound more tightly to

myosin at the lower pH, while SL-ADP bound about the same. This conclusion is also supported by the observation that the ratio of K_d^{app} (the concentration of SL-ADP required to half-saturate the tension response), to [ATP] is greater at the lower pH, see Discussion.

DISCUSSION

The main findings of the present study were that, SL-ADP increased isometric tension and decreased ATPase rate in skinned rabbit psoas muscle fibers. In current models of cross-bridge interaction, ATP is thought to dissociate the rigor state cross-bridges (nucleotide-free myosin heads) which occur upon release of ADP from force generating states in the cross-bridge cycle (17, 21, 36). The binding of ADP, preventing cross-bridge dissociation, *in vivo*, would be expected to increase tension and decrease ATP utilization rate. In general, as discussed in the introduction, experimental studies have confirmed these predictions as do the results presented here. The measurements described here have explored the effects of the competition of ATP with an ADP analog over a wider range of concentrations of both nucleotides, allowing us to show that the ADP analog is a pure competitor with ATP and to determine how this competition changes with changing conditions. In particular we address the question of the role of ADP in muscle fatigue. Finally we present a model that describes nucleotide competition in isometric fibers.

Muscle properties at pH 7. The current results support and extend the results of a number of previous investigations all of which showed that increased [ADP] potentiates isometric tension (8, 13, 27, 29, 30, 40, 23). In most of these investigations high concentrations of ADP competed against high concentrations of ATP in the absence of an ATP regenerating system. We have advanced these observations by showing that this effect is also seen over a wider range of nucleotide concentrations, including more physiological (lower) concentrations of ADP. The potentiation of tension measured here at these lower ADP concentrations is in rough agreement with that seen earlier when

the concentrations are extrapolated to the higher values previously used. We are in agreement with one study that showed that at high levels of ATP, low levels of ADP have little or no effect (4). We also agree with a study where addition of 0.7 mM ADP resulted in ~ 7% force increase (23). In the present study, the ATPase activity of isometric myofibrils was inhibited by SL-ADP. The values for K_m for ATP and for K_i, of 29 μM and 240 μM at pH 7, are both slightly higher than those observed by Sleep and co-workers, using ADP instead of SL-ADP, K_m = 17 μM and K_I = 170 μM (22, 39).

The effects of lower pH and/or increased P_i. At pH 6, a pH that is approached during severe fatigue, there was a decrement in tension relative to that found at pH 7 of approximately 50%, as it has previously been shown (5, 16, 23, 28, 31, 32). The addition of SL-ADP produced the same absolute increase in fiber tension as was found at pH 7 (Table 1 or Fig. 2), which a simple model, described below, could explain. K_d^{app} was about 2-3 fold higher, showing that SL-ADP competed more weakly with ATP, than at pH 7. We found that the weaker competition arises from tighter binding of ATP. At pH 6 we found a decrease in the value of K_m to 4.8 ± 2 μM, however the value of K_i remained the same (at 280 ± 50 μM), indicating that ATP bound more tightly to myosin at the lower pH, while SL-ADP bound about the same. No previous study has explored the effects of pH on nucleotide binding in fibers, and the tighter binding of ATP at the lower pH could have important implications for fatigue.

An increase in P_i at pH 7 lowered tension to about the same level as achieved at pH 6, but P_i also decreased the absolute increment in tension, P', produced by SL-ADP. Addition of P_i increased the value of K_d^{app}, indicating that SL-ADP competed more weakly with ATP at the higher P_i. This could be due to a direct competition between SL-

ADP and P_i for the active site of myosin. Previous work has shown a weak competition between ATP and P_i (35).

Fatigue. In general, during fatigue *in vivo*, average [ADP] is observed to rise to about 200 μM and average [ATP] to not fall below 2-3 mM see (20) for review. The effects of ADP seen *in vitro* in skinned fibers and myofibrils under similar conditions have been too small to produce a significant effect on the physiological properties of fatigued fibers *in vivo.* Our results generally agree with this conclusion. Our data using SL-ADP predict that if the ADP concentration was raised from 20 μM to 500 μM, and the ATP level was decreased from 5 mM to 2 mM, that the effect of ADP would be a 4% increase in force at pH 7. At pH 6 the relative effect of SL-ADP is greater, but it is a weaker competitor with ATP, so the relative effect on tension would be about the same as at pH 7. Thus the rise in ADP seen during fatigue can only play a significant role if the actual changes in the concentrations of the nucleotides are greater in the interior of the myofibrils than what has been inferred from measurements averaged over whole muscles. Is this possible? The ATP and ADP concentrations have been mostly measured in whole muscle using NMR or biopsy homogenates, (12, 20). Both of these methods produce an average value of each nucleotide distributed throughout the fiber and in many different fibers that may not be equally activated or equally fatigued. Recent analyses by one of us of single human muscle fibers using maximal intensity exercise (25, 26), have shown a large variability in nucleotide content of the different fiber types at rest and post-exercise; with much lower levels of ATP and near depletion of PCr in some post-exercise fast fiber fragments. Moreover, it has been calculated that ADP could rise to as high as 3.0 mM during contraction, when the PCr store is depleted

(43). Our measurements show that if myofibrillar ADP reached 1 mM with 3 mM ATP, the effect would be an approximately further 2-fold increase in force to about 8% at either pH 7 or pH 6. Although an increase in the ADP concentration may increase the tension economy by potentiating tension and inhibiting the ATPase activity, the effect on the tension, described above will still be modest, and the effect on ATPase activity will be even smaller. Thus another mechanism must also operate to produce the two-fold increase in tension economy seen *in vivo* fatigue (9, 10).

Modeling cross-bridge kinetics. Force and ATPase activity in isometric fibers as a function of ATP and SL-ADP were analyzed using a simple model to determine values for the relevant kinetic parameters governing the binding of nucleotides in the fiber, (Fig. 5). Cross-bridges are assumed to be in one of 4 states, described in Figure 5. The effect of SL-ADP is to increase the rate of the transition from the rigor state, 4, back into state 3. As the concentration of SL-ADP increases, the proportion of cross-bridges in state 4 decreases, effectively decreasing the rate of ATP binding by decreasing the concentration of state 4. The effect of increasing the rate of this transition will be to increase force, and inhibit the ATPase activity, as observed. The model allows us to quantify these phenomena and to draw connections between force and ATPase activity.

The goal of the model was to explain both the isometric force and the ATPase activity with the same set of rate constants and with the measured affinity of SL-ADP for the rigor state. This goal was not easily obtained, and a number of models were investigated that were not able to fit the data. The fit to the data was much worse in the absence of state 3, i.e. with the binding of SL-ADP leading directly back to the power stroke. Models in which the transitions from the non-force generating state 1, to the

force generating states 2 and 3 were effectively irreversible, all had a common problem. At saturating concentrations of SL-ADP all cross-bridges accumulated in state 3. Thus the force increase was greater when starting from conditions with lower force, modeled by a lower population of state 2, and the final force approached a maximum regardless of the initial conditions, in contrast to the data shown in figures 2 and 3. A variety of models in which additional states with bound SL-ADP had a different force per cross-bridge than that of active cross-bridges all suffered from a similar problem. This problem could be fixed by assuming that reverse transitions connect states 3 to 2 to 1, allowing the force generating states to exchange significantly with the pre-force state. Thus in the model, cross-bridges that bind SL-ADP can back up all the way to the pre-force state, which precedes the power stroke. This is a dramatic conclusion with a quasi equilibrium that spans the entire power stroke. In the model there is only one irreversible transition, that from state 4 back to state 1, a transition involving the binding of ATP. Measurements of the relative affinities of actin and ATP for myosin suggest that this transition should be irreversible (see (6) for review).

The model simulation of the ATPase activity as a function of [ATP] can be used to determine the rate constant for the binding of ATP to state 4, as described in methods. The observed value of K_m at pH 7 sets the second order binding constant for ATP to be 3.6×10^4 M^{-1} s^{-1}. Thus $k_{41} = 3.6 \times 10^4 \times [ATP]$ s^{-1}. The observed affinity of SL-ADP for myosin in rigor muscle K_d = 200 μM, measured using EPR spectroscopy was used to set the ratio of k_{34}/k_{43} ((11) and recent unpublished observations). Taking the value of k_{34} to be 50 s^{-1} thus $k_{43} = 2.5 \times 10^5 \times [ADP]$ s^{-1}. At pH 6 the lower value of K_m, 4.8 μM leads to a faster rate for the binding of ATP, of 8.7×10^4 M^{-1} s^{-1}. EPR

spectroscopy showed that the dissociation constant for SL-ADP binding to rigor fibers was not greatly changed by lowering the pH to 6, so the value of k_{43} remained as set at pH 7. The value for the second order binding constant for ATP, 3.6 x 10^4 M^{-1} s^{-1} at pH 7, is about 10 fold slower than that measured for binding to rigor fibers following photo-release of ATP from caged ATP (29). This may indicate that ATP binds more slowly to nucleotide free myosin heads in active fibers than in rigor fibers. The rate of binding of ADP or SL-ADP to either active or rigor cross-bridges has not been measured directly.

One test of the model is whether the second order rate constants, for the binding of nucleotides, discussed above, can accurately simulate the general properties of the effect of ATP and SL-ADP on isometric tension. We first consider the effect of ATP on fiber tension. As observed previously as [ATP] is raised from 50 μM to 1 mM the isometric tension decreases by about 30 - 40%. The most obvious explanation for the higher tension seen at the lower [ATP] is that it is due to the buildup of force generating cross-bridges in rigor states. However, the K_m for the ATPase activity observed here or earlier (22), 15-30 μM, suggested that rigor states would only be populated at ATP concentrations below 50 μM, leading some investigators to question this explanation (22). The model proposed here provides an excellent fit to the tension as a function of ATP using the observed value of K_m found for the ATPase activity. The apparent discrepancy between the low value for K_m and the range of [ATP] over which tension falls is resolved because the fraction of detached cross-bridges is large at all [ATP], allowing modest changes in k_{41} •[ATP] to provide significant changes in tension.

The model also explains the major features of the effect of SL-ADP on fiber tension. As [ATP] increases the concentration of SL-ADP required to achieve half of the

force potentiation, K_d^{app}, also increases (Table 1). As shown in Figure 6, the simulated values of K_d^{app} match the observed values very well. A stringent test of the model is that the greater value of K_d^{app} seen at pH 6 is quantitatively explained by the changes observed in the values of K_m and K_i at this pH. The 6-fold change in K_m, coupled with a 2-fold decrease in the maximum ATPase activity and the almost unchanged value of K_d for binding of SL-ADP, show that the strength of the competition of SL-ADP with ATP will decrease by about a factor of 3, which is close to what is observed in the tension measurements.

The model also predicts that the potentiation of tension achieved at saturating SL-ADP, P', is approximately a constant for different initial values of [ATP] and pH. When the pH is decreased from seven to six, tension decreases by more than half. This could be due to a decrease in the force generated per cross-bridge or it could be due to a decrease in the population of force generating cross-bridges. Measurements of fiber stiffness, which also decreases, suggest the latter possibility. This can be simulated in the model by decreasing the rate of the transition from detached to force generating cross-bridges, k_{12}, resulting in a buildup of cross-bridges in state 1, with a decrease in force generating cross-bridges. The model, now predicts that the addition of saturating SL-ADP produces approximately the same absolute increase in tension. The model predicts an approximately constant value of P' as [ATP] is varied above 50 µM, but underestimates the value at 50 µM. The model predicts that the ATPase activity will be inhibited by SL-ADP following approximately competitive behavior with a K_i of 140 µM at pH 7. This is lower than that observed here for SL-ADP, 240 µM, but is not far from that previously observed for ADP (170 µM) by Sleep and Glyn (39).

Addition of phosphate (P_i) also decreases force production, but in contrast to a change in pH, the value of P' is also decreased. Higher P_i concentrations lower the free energy available from the hydrolysis of ATP and thus lower the free energy available to generate isometric force see (35) for review. The decrease in fiber force in our model is thus attributed to a lower force generated in states 2 and 3. With this assumption the values of P' are decreased by the same relative amount as did P_0, which is more than is observed, see Figure 3. The difference between model and data shows that the effect of P_i is more complex than can be explained with this simple model.

The assumption in our model that cross-bridges can back up from the end to the beginning of the power stroke has not been considered in most previous models, but there is some evidence supporting it (2, 3). In general, strongly bound cross-bridges require binding of ATP to be dissociated from actin. Transitions in the forward direction through the power stroke are much faster than the reverse transitions, or the reverse transitions occur only upon stretching the cross-bridges into strained positions. Thus the progression through the power stroke is effectively irreversible. Our model does not include strain dependence, raising the question of how cross-bridges can make transitions from state 3, at the end of the power stroke, back to state 1, at the beginning of the power stroke. Reversal back to state 1 would require resynthesis of ATP from ADP and P_i. The input of energy required would come from fluctuations in the relative positions of actin and myosin, which occur on a time scale that is rapid compared to the cross-bridge cycle (37). Moreover, rapid transitions between strongly attached and detached cross-bridges have been observed by measurements of stiffness in rapid length changes (3). Furthermore incorporation of P_i from the medium into ATP in active

muscle fibers shows that the transition from state 2 to state 1 can occur (2). Our conclusions can also help provide a molecular explanation for one recent model of cross-bridge function in which force was treated as a macroscopic thermodynamic variable (1).

Conclusion. The present data define the strength of the competition between ATP and an analog of ADP, SL-ADP, which binds to myosin similarly to ADP. SL-ADP is a weak competitive inhibitor of ATP binding. Although it has a greater relative effect at a lower pH, ATP binds more tightly at the lower pH so that the competition occurs at even greater concentrations of SL-ADP. Thus, although an increase in the ADP concentration may increase the tension economy by potentiating tension and inhibiting the ATPase activity, the effect on the tension will be modest and the effect on ATPase activity will be even smaller in fatigue. The simple model presented in Figure 5 correctly predicts many of the major features of the effect of addition of SL-ADP on the properties of isometric active fibers. A major conclusion is that the effects of SL-ADP on both tension and on ATPase activity can be explained with the same kinetic constants in a simple 4-state model. The agreement between model and data required that cross-bridges can make reverse transitions from the states at the end of the force generation back to non force generating states, which is a novel proposition.

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REFERENCES

1. Baker, J. E., and D. D. Thomas. Thermodynamics and kinetics of a molecular motor ensemble. *Biophys J* 79: 1731-1736, 2000.

2. Bowater, R., and J. Sleep. Demembranated muscle fibers catalyze a more rapid exchange between phosphate and ATP than actomyosin subfragment 1. *Biochem* 27: 5314-5323, 1988.

3. Brenner, B. Rapid dissociation and reassociation of actomyosin cross-bridges during force generation: a newly observed facet of cross-bridge action in muscle. *Proc Natl Acad Sci U S A* 88: 10490-10494, 1991.

4. Chase, P. B., and M. J. Kushmerick. Effect of physiological ADP concentrations on contraction of single skinned fibers from rabbit fast and slow muscles. *Am J Physiol* 268: C480-C489, 1995.

5. Chase, P. B., and M. J. Kushmerick. Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. *Biophys J* 53: 935-946, 1988.

6. Cooke, R. Actomyosin interaction in striated muscle. *Physiol Rev* 77: 671-697, 1997.

7. Cooke, R., K. Franks, G. Lucianni, and E. Pate. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J Physiol (Lond)* 395: 77-97, 1988.

8. Cooke, R., and E. Pate. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys J* 48: 789-798, 1985.

9. Crow, M. T., and M. J. Kushmerick. Correlated reduction of velocity of shortening and the rate of energy utilization in mouse fast-twitch muscle during a continuous tetanus. *J Gen Physiol* 82: 703-720, 1983.

10. Crow, M. T., and M. J. Kushmerick. Myosin light chain phosphorylation is associated with a decrease in the energy cost for contraction in fast twitch mouse muscle. *J Biol Chem* 257: 2121-2124, 1982.

11. Crowder, M. S., and R. Cooke. Orientation of spin-labeled nucleotides bound to myosin in glycerinated muscle fibers. *Biophys J* 51: 323-33, 1987.

12. Dahlstedt, A. J., A. Katz, B. Wieringa, and H. Westerblad. Is creatine kinase responsible for fatigue? Studies of isolated skeletal muscle deficient in creatine kinase. *FASEB J* 14: 982-990, 2000.

13. Dantzig, J. A., M. G. Hibberd, D. R. Trentham, and Y. E. Goldman. Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibres. *J Physiol (Lond)* 432: 639-680, 1991.

14. Dawson, M. J., D. G. Gadian, and D. R. Wilkie. Muscular fatigue investigated by phosphorus nuclear magnetic resonance. *Nature* 274: 861-866, 1978.

15. de Haan, A., D. A. Jones, and A. J. Sargeant. Changes in velocity of shortening, power output and relaxation rate during fatigue of rat medial gastrocnemius muscle. *Pflugers Arch* 413: 422-428, 1989.

16. Edman, K. A., and A. R. Mattiazzi. Effects of fatigue and altered pH on isometric force and velocity of shortening at zero load in frog muscle fibres. *J Muscle Res Cell Motil* 2: 321-334, 1981.

17. Eisenberg, E., T. L. Hill, and Y. Chen. Cross-bridge model of muscle contraction. *Biophys. J.* 29: 195-227, 1980.

18. Etlinger, J. D., R. Zak, and D. A. Fischman. Compositional studies of myofibrils from rabbit striated muscle. *J Cell Biol* 68: 123-141, 1976.

19. Ferenczi, M. A., E. Homsher, and D. R. Trentham. The kinetics of magnesium adenosine triphosphate cleavage in skinned muscle fibres of the rabbit. *J Physiol (Lond)* 352: 575-599, 1984.

20. Fitts, R. H. Muscle fatigue: the cellular aspects. *Am J Sports Med* 24: S9-13, 1996.

21. Geeves, M. A. The dynamics of actin and myosin association and the cross bridge model of muscle contraction. *Biochem J* 274: 1-14, 1991.

22. Glyn, H., and J. Sleep. Dependence of adenosine triphosphatase activity of rabbit psoas muscle fibres and myofibrils on substrate concentration. *J Physiol (Lond)* 365: 259-276, 1985.

23. Godt, R. E., and T. M. Nosek. Changes of intracellular milieu with fatigue or hypoxia depress contraction of skinned rabbit skeletal and cardiac muscle. *J Physiol (Lond)* 412: 155-180, 1989.

24. Hatcher, D. D., and A. R. Luff. Contractile properties of cat skeletal muscle after repetitive stimulation. *J Appl Physiol* 64: 502-510, 1988.

25. Karatzaferi, C., A. de Haan, R. A. Ferguson, W. van Mechelen, and A. J. Sargeant. Phosphocreatine and ATP content in human single muscle fibres before and after maximum dynamic exercise. *Pflugers Arch* 442: 467-474, 2001.

26. Karatzaferi, C., A. de Haan, W. van Mechelen, and A. J. Sargeant. Metabolic changes in single human fibres during brief maximal exercise. *Exp Physiol* 86: 411-415, 2001.

27. Kawai, M., and H. R. Halvorson. Role of MgATP and MgADP in the cross-bridge kinetics in chemically skinned rabbit psoas fibers. Study of a fast exponential process (C). *Biophys J* 55: 595-603, 1989.

28. Kentish, J. C., and S. Palmer. Effect of pH on force and stiffness in skinned muscles isolated from rat guinea-pig ventricle and from rabbit psoas muscle. *J. Phys.* 410: 67P, 1988.

29. Lu, Z., R. L. Moss, and J. W. Walker. Tension transients initiated by photogeneration of MgADP in skinned skeletal muscle fibers. *J Gen Physiol* 101: 867-888, 1993.

30. Metzger, J. M. Effects of phosphate and ADP on shortening velocity during maximal and submaximal calcium activation of the thin filament in skeletal muscle fibers. *Biophys J* 70: 409-417, 1996.

31. Metzger, J. M., and R. L. Moss. Effects of tension and stiffness due to reduced pH in mammalian fast- and slow-twitch skinned skeletal muscle fibres. *J Physiol (Lond)* 428: 737-750, 1990.

32. Myburgh, K. H., and R. Cooke. Response of compressed skinned skeletal muscle fibers to conditions that simulate fatigue. *J Appl Physiol* 82: 1297-1304, 1997.

33. Myburgh, K. H., K. Franks-Skiba, and R. Cooke. Nucleotide turnover rate measured in fully relaxed rabbit skeletal muscle myofibrils. *J Gen Physiol* 106: 957-973, 1995.

34. Pate, E., M. Bhimani, K. Franks Skiba, and R. Cooke. Reduced effect of pH on skinned rabbit psoas muscle mechanics at high temperatures: implications for fatigue. *J Physiol (Lond)* 486: 689-694, 1995.

35. Pate, E., and R. Cooke. Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke. *Pflugers Arch* 414: 73-81, 1989.

36. Pate, E., and R. Cooke. A model of cross bridge action: the effects of ATP, ADP and P_i. *J Muscle Res Cell Motil* 10: 181-196, 1989.

37. Pate, E., and R. Cooke. Simulation of stochastic processes in motile cross bridge systems. *J Muscle Res Cell Motil* 12: 376-393, 1991.

38. Siemankowski, R. F., M. O. Wiseman, and H. D. White. ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proc Natl Acad Sci U S A* 82: 658-662, 1985.

39. Sleep, J., and H. Glyn. Inhibition of myofibrillar and actomyosin subfragment 1 adenosinetriphosphatase by adenosine 5'-diphosphate, pyrophosphate, and adenyl-5'-yl imidodiphosphate. *Biochem* 25: 1149-1154, 1986.

40. Wang, G., and M. Kawai. Effects of MgATP and MgADP on the cross-bridge kinetics of rabbit soleus slow-twitch muscle fibers. *Biophys J* 71: 1450-1461, 1996.

41. Westerblad, H., D. G. Allen, J. D. Bruton, F. H. Andrade, and J. Lannergren. Mechanisms underlying the reduction of isometric force in skeletal muscle fatigue. *Acta Physiol Scand* 162: 253-260, 1998.

42. Westerblad, H., J. Bruton, and J. Lannergren. The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature. *J Physiol (Lond)* 500: 193-204, 1997.

43. Westerblad, H., and J. Lannergren. Reduced maximum shortening velocity in the absence of phosphocreatine observed in intact fibres of Xenopus skeletal muscle. *J Physiol (Lond)* 482: 383-390, 1995.

44. Wiseman, R. W., T. W. Beck, and P. B. Chase. Effect of intracellular pH on force development depends on temperature in intact skeletal muscle from mouse. *Am J Physiol* 271: C878-886, 1996.

45. Woodward, S. K., J. F. Eccleston, and M. A. Geeves. Kinetics of the interaction of 2'(3')-O-(N-methylanthraniloyl)-ATP with myosin subfragment 1 and actomyosin subfragment 1: characterization of two acto-S1-ADP complexes. *Biochemistry* 30: 422-430, 1991.

Table 1. Maximal tension (P_0) as mean ± SD, tension increase (P') and kinetic parameters (± 95% confidence), all as a function of SL-ADP at 10 ^{o}C, for n=3 -7 fibers per condition, (variable pH, [P_i] or [ATP]). P' values and kinetic parameters are based on fits (see text) on averaged data points.

pH	[P_i]	[ATP]	P_0	P'	K_d^{app}	K_d^{app}/[ATP]
	mM	mM	N.mm^{-2}	N.mm^{-2}	mM	mM
7	3.0	0.050	0.120 ±0.005	0.045 ± 0.001	0.10 ± 0.008	2.00
7	3.0	0.150	0.095 ±0.0069	0.039 ± 0.002	0.29 ± 0.053	1.93
7	3.0	0.500	0.082 ±0.003	0.051 ± 0.003	1.63 ± 0.223	3.26
7	10	0.150	0.070 ±0.0107	0.030 ±0.0006	0.49 ± 0.040	3.27
7	54	0.150	0.019 ±0.0054	0.019 ±0.0008	0.93 ± 0.11	6.20
6	3.0	0.150	0.032 ±0.001	0.044 ±0.004	1.05 ± 0.227	7.00
6	10	0.150	0.014 ±0.0027	0.029 ±0.006	1.13 ± 0.526	7.53

FIGURE LEGENDS

Figure 1. Fiber tension is shown as a function of time. The fiber was initially in a relaxing solution in the presence of 50 μM ATP, and at 3 seconds calcium was added to activate the fiber. Following mixing, 500 μM SL-ADP was added which caused an increase in isometric tension of ~ 35%. Data were obtained from a single psoas fiber with a diameter of 60 μm.

Figure 2. Fiber tension (mean ± SD) is shown as a function of the SL-ADP concentration, for different values of the ATP concentration and at two different values of pH, for n=3 -7 fibers per condition. Data were obtained as outlined in Figure 1. The solid lines represent a fit to each data set, assuming that the force increase could be described as a simple binding isotherm, as described by Equation 1 in the text.

Figure 3. Fiber tension (mean ± SD) is shown as a function of the SL-ADP concentration for different values of phosphate (P_i) concentration at pH 7 and 6, for n=3 -7 fibers per condition. All data were obtained using 150 μM ATP. The solid lines represent a fit to each data set, assuming that the force increase could be described as a simple binding isotherm, as described by Equation 1 in the text.

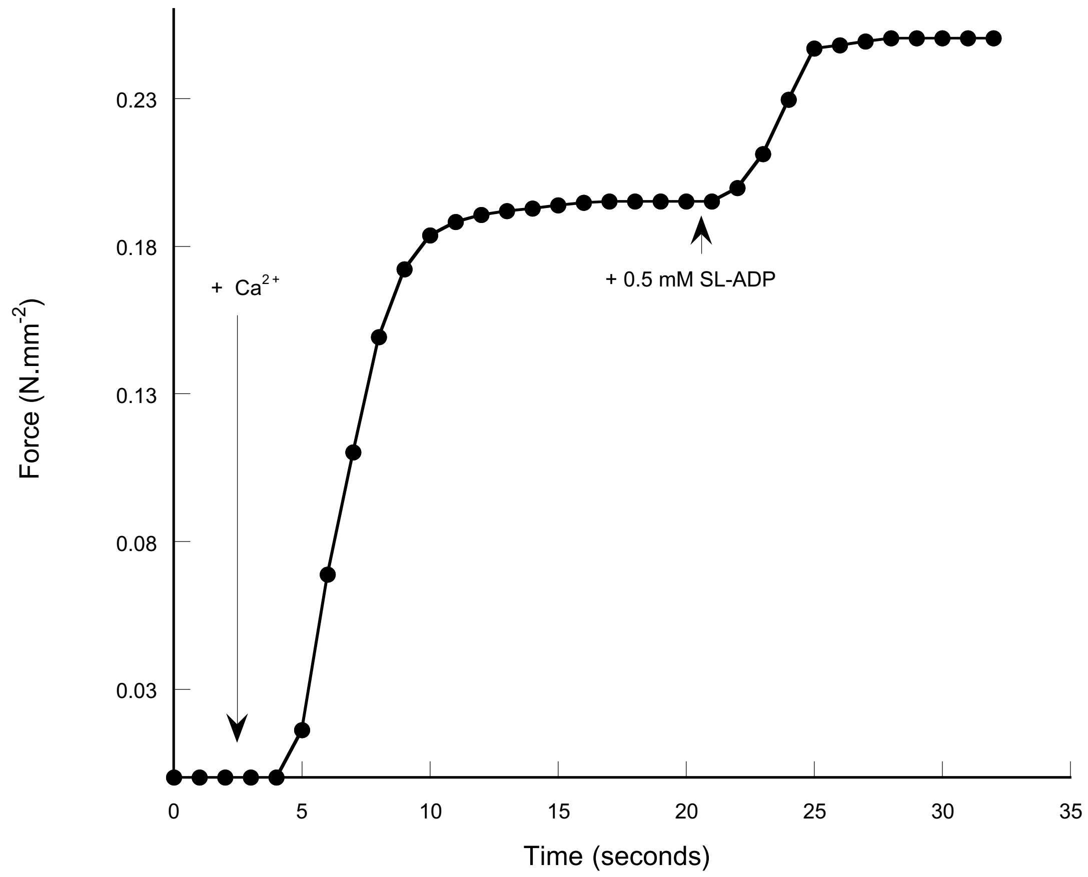
Figure 4. Double reciprocal plots of the ATPase activity of fully activated myofibrils as a function of [ATP] with (filled symbols) or without (open symbols) SL-ADP at pH 7 (panel A) and 6 (panel B). The ATPase activity was determined by use of an enzymatic system to link the production of ADP to NADH as described in Methods. ■ 2mM SL-

SLADP; ● 0.5 mM SL-SLADP; ○ Control; □ Control with double PK. Panel **A**: pH 7. The linear fits for pH7 define: Control: V_{max} = 0.95 s^{-1}, K_m for ATP = 28 μM; Control 2 x PK: V_{max} = 1.1 s^{-1}, K_m for ATP = 29 μM; 0.5 mM SL-ADP: V_{max} = 0.84 s^{-1}, K_m for ATP = 94 μM, K_i = 220 μM; 2 mM SL-ADP: V_{max} = 0.82 s^{-1}, K_m for ATP = 241 μM, K_i = 260 μM. Panel **B**: pH 6. The linear fits for pH 6 define: Control: V_{max} = 0.42 s^{-1} K_m for ATP = 4.8 μM; 2 mM SL-ADP: V_{max} = 0.0.62 s^{-1}, K_m for ATP = 39 μM, and a K_i for SL-ADP = 280 μM.

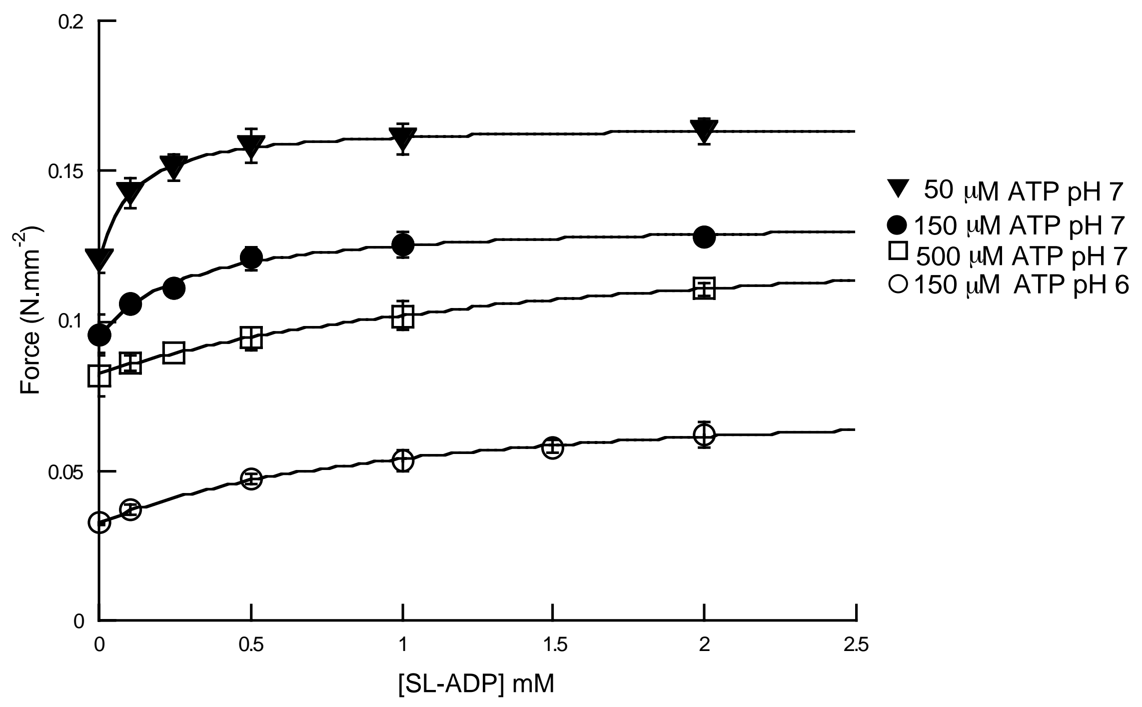
Figure 5. Force and ATPase activity in isometric fibers as a function of ATP and SL-ADP were analyzed using a simple model to determine values for the relevant kinetic parameters governing the binding of nucleotides in the fiber. Cross-bridges are assumed to be in one of 4 states: (1) detached from actin, or attached weakly; (2) attached to actin in the power stroke; (3) state reached by binding SL-ADP; (4) attached to actin in rigor. States 2, 3 and 4 all generate force. The effect of SL-ADP is to compete with ATP for binding to state 4. Cross-bridges in state 1 make transitions with a first order rate constant k_{12} to state 2, where they are strongly bound to actin and generate force. Cross-bridges in state 4 can either bind to ATP in a second order reaction with rate constant k_{41} leading back to state 1 and continuing the cycle, or they can bind to SL-ADP leading back to state 3. The model was solved to provide values of force and ATPase activity, as described in the Methods. The constant value of P', the force increase at saturating SL-ADP, could be fit by assuming that the reverse transitions between states 1 to 4 were fast, allowing the force generating states to

“effectively equilibrate” with the pre-force state The model was analyzed using Berkeley Madonna, as described in Methods.

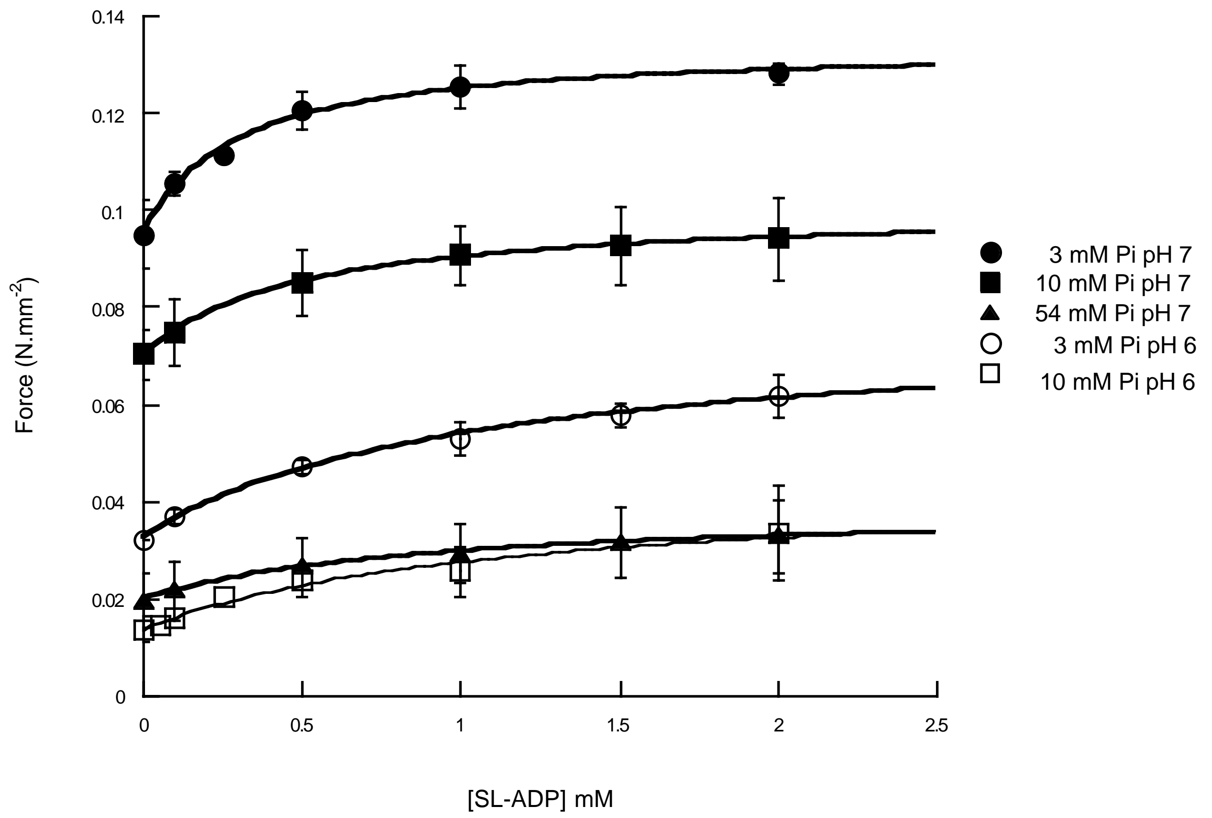
Figure 6. The simulated values of tension, solid lines, are compared to the data shown in Figure 2. The rate constant for binding ATP was determined from a fit to the ATPase activity as a function of ATP. The rate constant for binding SL-ADP was taken from its measured affinity for rigor fibers. The values of the other rate constants are given in Methods.



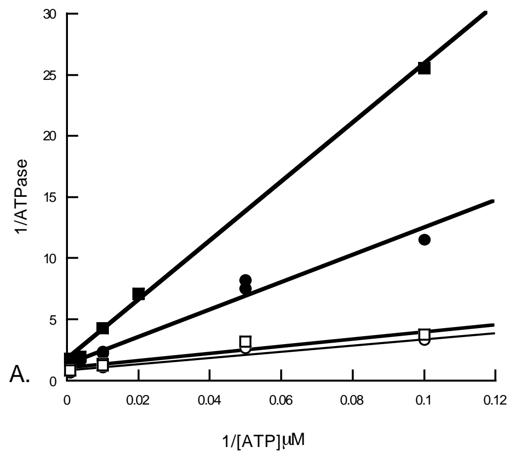
Force (N.mm^{-2})
0.23
0.18
0.13
0.08
0.03
+ Ca^{2+}
+ 0.5 mM SL-ADP
0
5
10
15
20
25
30
35
Time (seconds)

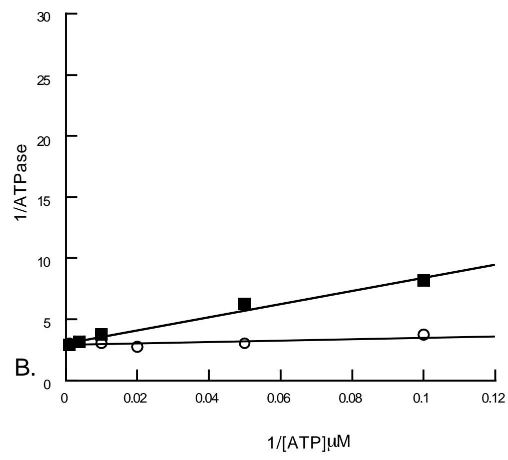


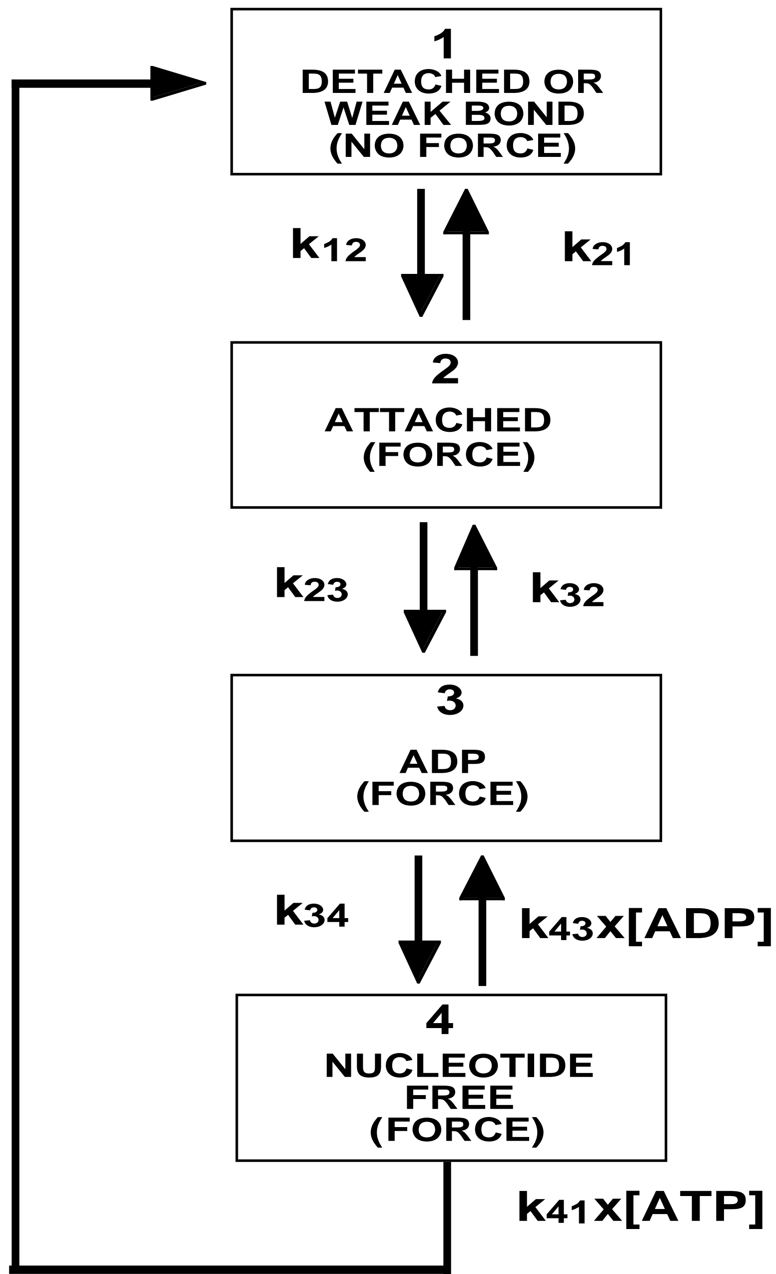
Force (N.mm⁻²)
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
[SL-ADP] mM
50 μM ATP pH 7
150 μM ATP pH 7
500 μM ATP pH 7
150 μM ATP pH 6

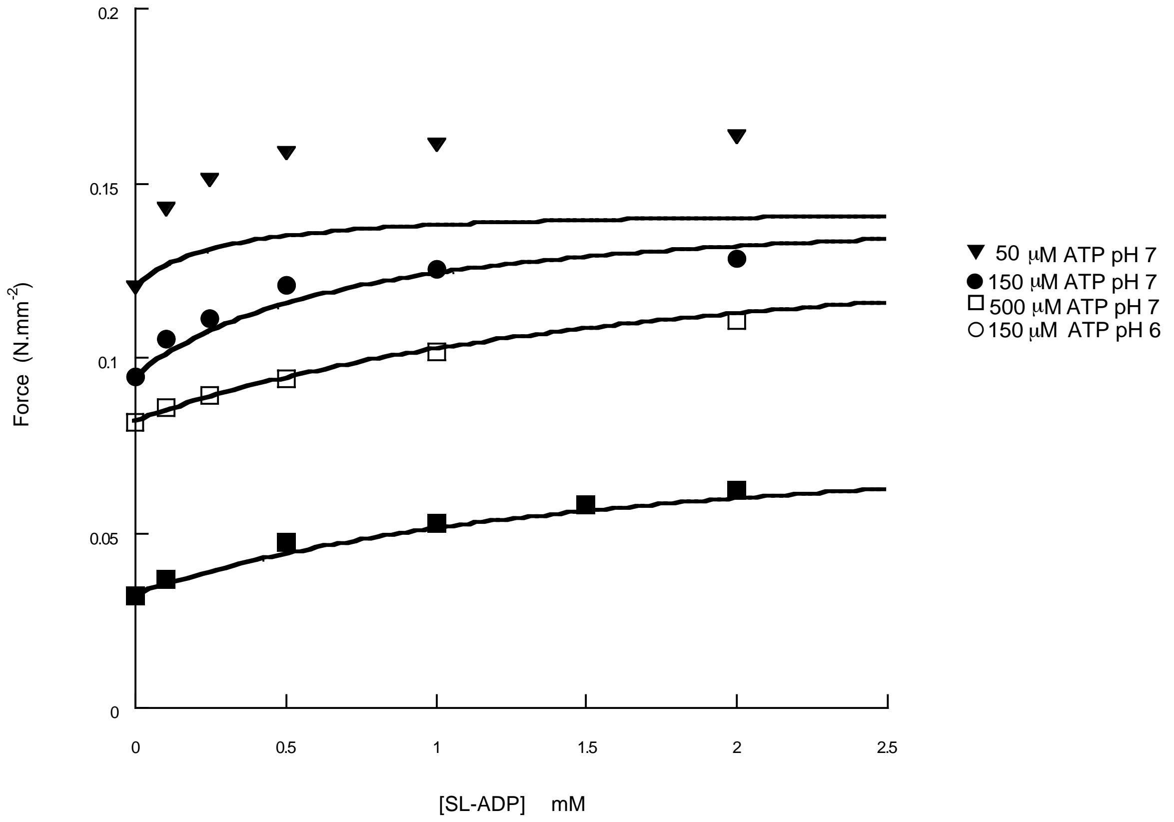


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Force (N.mm-2)
0
0.5
1
1.5
2
2.5
[SL-ADP] mM
3 mM Pi pH 7
10 mM Pi pH 7
54 mM Pi pH 7
3 mM Pi pH 6
10 mM Pi pH 6


A.
1/ATPase
1/[ATP]μM
0
5
10
15
20
25
30
0
0.02
0.04
0.06
0.08
0.1
0.12


B.
1/ATPase
1/[ATP]μM
0
5
10
15
20
25
30
0
0.02
0.04
0.06
0.08
0.1
0.12


1
DETACHED OR
WEAK BOND
(NO FORCE)
k12
k21
2
ATTACHED
(FORCE)
k23
k32
3
ADP
(FORCE)
k34
k43x[ADP]
4
NUCLEOTIDE
FREE
(FORCE)
k41x[ATP]


0.2
0.15
0.1
0.05
0
Force (N.mm-2)
0
0.5
1
1.5
2
2.5
[SL-ADP] mM
50 μM ATP pH 7
150 μM ATP pH 7
500 μM ATP pH 7
150 μM ATP pH 6