

Characterizations of Cross-Bridges in the Presence of Saturating Concentrations of MgAMP-PNP in Rabbit Permeabilized Psoas Muscle

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Several earlier studies have led to different conclusions about the complex of myosin with MgAMP-PNP. It has been suggested that subfragment 1 of myosin (S1)-MgAMP-PNP forms an S1-MgADP-like state, an intermediate between the myosin S1-MgATP and myosin S1-MgADP states or a mixture of cross-bridge states. We suggest that the different states observed result from the failure to saturate S1 with MgAMP-PNP. At saturating MgAMP-PNP, the interaction of myosin S1 with actin is very similar to that which occurs in the presence of MgATP. 1) At 1°C and 170 mM ionic strength the equatorial x-ray diffraction intensity ratio I_{11}/I_{10} decreased with an increasing MgAMP-PNP concentration and leveled off by ~20 mM MgAMP-PNP. The resulting ratio was the same for MgATP-relaxed fibers. 2) The two dimensional x-ray diffraction patterns from MgATP-relaxed and MgAMP-PNP-relaxed bundles are similar. 3) The affinity of S1-MgAMP-PNP for the actin-tropomyosin-troponin complex in solution in the absence of free calcium is comparable with that of S1-MgATP. 4) In the presence of calcium, I_{11}/I_{10} decreased toward the relaxed value with increasing MgAMP-PNP, signifying that the affinity between cross-bridge and actin is weakened by MgAMP-PNP. 5) The degree to which the equatorial intensity ratio decreases as the ionic strength increases is similar in MgAMP-PNP and MgATP. Therefore, results from both fiber and solution studies suggest that MgAMP-PNP acts as a non hydrolyzable MgATP analogue for myosin.

INTRODUCTION

During the hydrolysis of MgATP by myosin, various intermediate states are produced that have their own distinct properties with respect to their interactions with actin and regulated actin (actin-tropomyosin-troponin). The correlation of particular biochemical states with mechanical and structural states occurring during muscle contraction has relied on the use of stable nucleotide analogues, which mimic different intermediate states. This is particularly true for ATP-like analogues since hydrolysis of ATP in vivo leads to a distribution of states.

Myosin-nucleotide complexes may be broadly grouped into two types of chemical states although there are variations within each group (see Chalovich, 1992 for a more complete survey of these states). Myosin that has MgADP or no bound nucleotide has several distinguishing features. Its binding to actin is strong (Highsmith, 1990), its dissociation from actin is relatively slow in solution (White and Taylor, 1976; Marston, 1982; Konrad and Goody, 1982) and in fibers (Schoenberg and Eisenberg, 1985), and its binding to regulated actin is inhibited in the absence of Ca^{2+} and increases cooperatively as the S1 concentration is increased (Greene and Eisenberg, 1980). In addition, the binding of myosin in these states to regulated actin can

stabilize the active state of actin even under relaxing conditions (Bremel and Weber, 1972). Such states are often called strong binding or activating.

When myosin contains bound MgATP (or ADP-P_i), the characteristics of the cross-bridges are different. These states, which are mostly observed in relaxed muscle fibers, are characterized by rapid attachment/detachment with actin (Lymn and Taylor, 1971; Stein et al., 1979), acto-myosin binding is not inhibited in the absence of Ca^{2+} (Chalovich and Eisenberg, 1982), does not cause appreciable activation of regulated actin (Chalovich et al., 1983), and gives rise to stiffness in relaxed muscle fiber preparations (Brenner et al., 1982; Schoenberg et al., 1984). The weak binding between actin and myosin can also be observed in both solution and in fibers by fluorescent probes placed on TnI (Trybus and Taylor, 1980) and by the effect of caldesmon on this interaction (Brenner et al., 1991; Kraft et al., 1995). These states, which have properties similar to those described for S1-MgATP in solution, are often called weak binding states or nonactivating states.

Several compounds have been used to stabilize the non-activating state of S1 including MgATP γ S (Dantzig et al., 1988; Kraft et al., 1992) and MgGTP (Frisbie et al., 1997a). With these and other substrates however, the problem of hydrolysis and rapid release of nucleotides in the presence of actin is not totally eliminated. MgAMP-PNP (Yount et al., 1991a,b) has been used widely in the past with different muscle sources including rabbit psoas muscle (Marston et al., 1976; Lymn, 1975; Schoenberg 1993; Berger and Thomas, 1994), insect flight muscle (Goody et al., 1975; Reedy et al., 1983, 1987), and frog sartorius muscle (Padron et al., 1984). In rabbit skeletal muscle, MgAMP-PNP has been thought to cause activating states (e.g., Schoenberg,

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Abbreviations used: AMP-PNP, 5'-adenylylimido-diphosphate.

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1993; Berger and Thomas, 1994), nonactivating states (e.g., Yount et al., 1971a,b), or a mixture of cross-bridge states (Lyman, 1975; Marston et al., 1976; Padron et al., 1984; Fajer et al., 1988). We have used several criteria both in solution and in fibers to characterize the state of the myosin-MgAMP-PNP. Our current findings indicate that in rabbit psoas muscle with saturating concentrations of MgAMP-PNP, high ionic strength, and low temperature, myosin interacts with actin as though the cross-bridges were in a weak binding/nonactivating state. Preliminary results have been presented previously (Frisbie and Yu, 1995; Frisbie et al., 1997b).

MATERIALS AND METHODS

Fiber preparation

Single skinned fibers of rabbit psoas muscle that were used in x-ray equatorial diffraction studies were prepared and mounted as previously described (Brenner et al., 1982; Yu and Brenner, 1989). The membranes were made permeable by using the skinning solution listed below. Single fibers were prepared ~3–4 h after the sacrifice of the rabbit. Only fresh fibers, which were less than 5 days old, were used. Sarcomere length was 2.35 μm . The temperature was maintained at 1°C during x-ray diffraction experiments. Histoacryl blue from B. Braun Melsungen AG (Melsungen, Germany) was used to glue the ends of each fiber to the carbon tips in the specimen chamber used in the x-ray diffraction setup.

For two-dimensional x-ray diffraction studies, muscle bundles were prepared using the same solutions as for the single fibers. Each bundle contained ~30 fibers.

Solutions

Fresh rabbit psoas muscle fibers were skinned using a solution of 0.5% Triton X-100, 5 mM KH_2PO_4 , 3 mM Mg acetate, 5 mM EGTA¹, 3 mM ATP, 50 mM creatine phosphate, 5 mM sodium azide, 1 mM dithiothreitol, 0.01 mM leupeptin, 0.001 mg/ml aprotinin, 0.01 mM antipain, 0.01 mM trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (Calbiochem–Novabiochem, La Jolla, CA), 0.001 mM pepstatin, and 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. The solutions used for x-ray diffraction experiments contained 10 mM imidazole, 2 mM MgCl_2 , 1 mM EGTA (or CaEGTA for solutions with a high calcium concentration), 1 mM dithiothreitol, and 0.2 mM P1-P5-di-adenosine-5-pentaphosphate (Sigma, St. Louis, MO) plus the various concentrations of MgAMP-PNP. The ionic strength was maintained at 170 mM by adding the appropriate amount of potassium propionate. P1-P5-di-adenosine-5-pentaphosphate was added to prevent myokinase from synthesizing ATP from endogenous ADP. Hexokinase was added to convert any endogenous ATP to ADP. When 0–1 mM MgAMP-PNP was used, 1 unit/ml hexokinase and 50 mM glucose were added. When the MgAMP-PNP concentration was in the 5–40 mM range, hexokinase was 3 units/ml, and glucose was 60 mM. The rigor solutions were identical to the MgAMP-PNP solutions but did not contain nucleotide. The 20 mM ionic strength relaxing solution was 10 mM in imidazole, 2 mM in MgCl_2 , 1 mM in NaEGTA, and 1 mM in MgATP. For 170 mM ionic strength relaxing solution higher concentrations of MgATP were used (2 mM for single fibers, 10 mM for bundles). Ionic strength was adjusted by potassium propionate.

Measurement of the association constant of actin-troponin-tropomyosin binding to S1-MgAMP-PNP

The association constant of S1-MgAMP-PNP binding to the regulated actin filament in EGTA solution was determined as described previously at 25°C

(Chalovich and Eisenberg, 1982) except for the modifications described below. A solution containing 20 mM MgAMP-PNP was used for determination of the association constant. This was diluted to 1 mM MgAMP-PNP using a solution containing 10 mM imidazole and 1 mM dithiothreitol before the MgATPase assays were performed to measure unbound S1. This dilution of the MgAMP-PNP minimized the competition between MgATP and the MgAMP-PNP.

Purity and ionic strength of MgAMP-PNP solutions

Since a high concentration of nucleotide was used in our experiments, the nucleotide accounts for a large percentage of the total ionic strength of the solution. The conductivities of the MgAMP-PNP containing solutions were matched to the conductivities of a series of MgATP-containing solutions measured at the same concentrations of nucleotide. The conductivity of potassium chloride was used as a standard. It was assumed that the mobilities of MgATP ions and the MgAMP-PNP ions were similar. Each 1-mM MgAMP-PNP was found to contribute an equivalent of 3 mM to the ionic strength of the solution. The difference between the ionization of MgATP and MgAMP-PNP can be partially attributed to the pKa being 7.7 ± 0.1 for the γ -phosphate of the MgAMP-PNP compared with 7.1 for MgATP (Yount et al., 1971a,b). The final ionic strength (170 mM) was adjusted by adding appropriate amounts of potassium propionate to a series of concentrations of MgAMP-PNP.

Purity of AMP-PNP was checked by a high performance liquid chromatography (HPLC) Phenomenex (Torrance, CA) Primesphere 5-mm C18 HC 250 \times 4.6-mm HPLC column using a Hewlett Packard (Wilmington, DE) 1090 System to determine possible impurities. A major contaminant was found, especially after purification on Sephadex A25. This contaminant ranged from ~5% from a freshly opened bottle to ~25% after purification and was identified as the degradation product AMP-PN using the method of Yount et al. (1971a,b). Briefly, the procedure was as follows: *Escherichia coli* alkaline phosphatase (Sigma) and *Crotalus adamanteus* phosphodiesterase I (Sigma) were used to enzymatically breakdown MgAMP-PNP to ADP-NH₂ and AMP + PNP_i, respectively. The reactions were carried out for a total of 16 h. Aliquots were taken at various time points and applied to Whatman No. 31ET paper for thin layer chromatography. Two solvent systems were used. The first consisted of a 20:20:20:39:1 ratio of 2-propanol/dimethylformamide/methyl ethyl ketone/water/concentrated ammonia. The second solvent system consisted of a 6:3:1 ratio of 1-propanol/concentrated ammonia/water. Adenosine containing spots were detected using a UV transilluminator. Phosphorus-containing spots were detected by first exposing the paper to acid hydrolysis by suspending it over boiling 4N HCL for ~45 min and then spraying the chromatogram with an acid molybdate spray reagent from Sigma. Phosphorus-containing spots turned blue after reacting with the spray reagent.

Due to degradation of the MgAMP-PNP during purification, perhaps due to thermal breakdown during lyophilization, unpurified MgAMP-PNP from newly opened bottles was used for x-ray diffraction and biochemical experiments. An extinction coefficient $\epsilon_{260\text{nm}}^{\text{mM}}$ of 13,300 was used to determine the concentration of MgAMP-PNP.

The possibility of MgATP contamination and its effect on the detachment of cross-bridges was examined by solving a series of simultaneous equations using the program Mathematica (Wolfram Research Inc., Champagne, IL). The equilibrium constants used in the equations are given in Table 1. The concentrations of pertinent protein complexes were calculated if 0.5 μM contaminating ATP was present. This concentration was chosen since it is the lower limit of this nucleotide that can be detected by HPLC in the presence of AMP-PNP.

X-ray diffraction studies

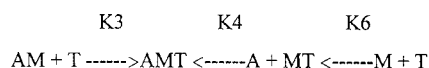
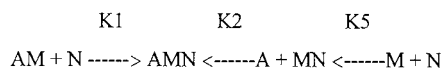
Equatorial patterns from single fibers

Equatorial x-ray diffraction experiments on single skinned fibers were carried out as described previously (Brenner et al., 1984). The integrated

TABLE 1 Calculated Concentrations of Various Actin and Myosin Complexes by Mathematica

Input values				
(Actin)	3.0×10^{-4} M	5.0×10^{-5} M	5.0×10^{-4} M	1.0×10^{-3} M
(Myosin)	5.0×10^{-6} M	5.0×10^{-6} M	5.0×10^{-6} M	5×10^{-6} M
(AMP-PNP) _{TOTAL}	20 mM	20 mM	20 mM	0
(ATP) _{TOTAL}	0.5 μ M	0.5 μ M	0.5 μ M	1.0 μ M
Calculated values				
(MN)	4.2×10^{-6} M	4.6×10^{-6} M	3.9×10^{-6} M	0
(MT)	3.1×10^{-7} M	3.4×10^{-7} M	2.9×10^{-7} M	3.6×10^{-6} M
(AM)	2.5×10^{-11} M	4.6×10^{-12} M	3.9×10^{-11} M	1.4×10^{-13} M
(AMN)	5.0×10^{-7} M	9.1×10^{-8} M	7.8×10^{-7} M	0
(AMT)	3.7×10^{-8} M	6.9×10^{-9} M	5.8×10^{-8} M	1.4×10^{-6} M
(M) _{FREE}	2.1×10^{-9} M	2.3×10^{-9} M	1.9×10^{-10} M	3.6×10^{-12} M
(ATP) _{FREE}	1.5×10^{-7} M	1.5×10^{-7} M	1.5×10^{-7} M	1.0×10^{-3} M

Abbreviations used: M, myosin; A, actin; N, MgAMP-PNP; T, MgATP. Association constants: $K_1 = 1 \times 10^6 \text{ M}^{-1}$; $K_2 = 4 \times 10^2 \text{ M}^{-1}$; $K_3 = 1 \times 10^{10} \text{ M}^{-1}$; $K_4 = 4 \times 10^2 \text{ M}^{-1}$; $K_5 = 1 \times 10^6 \text{ M}^{-1}$; $K_6 = 1 \times 10^{10} \text{ M}^{-1}$.



equatorial intensities were determined using the MLAB curve-fitting program from Civilized Software, Inc. (Bethesda, MD). Exposures for MgATP-relaxed muscle fibers at $\mu = 20$ and 170 mM were obtained first, followed by patterns in rigor at $\mu = 170$ mM in CaEGTA and EGTA-containing solutions. MgAMP-PNP was added up to 20 mM for EGTA containing solutions and 40 mM for CaEGTA containing solutions in a stepwise fashion. CaEGTA- and EGTA-containing solutions were alternated to take into account differences in muscle position within the beam and to be able to compare the two titration curves directly. The entire series for each muscle fiber from rigor to a high concentration of MgAMP-PNP was collected on a single spot in order to minimize noise during acquisition. Since muscle fibers differ structurally in size and shape from one place to another, this form of data collection resulted in more consistent patterns and allowed exposure times of only 100 s per data point to be used. Low exposure times also minimized sample damage due to the harmful effects of the x-ray beam.

After muscle fibers were exposed to 40 mM MgAMP-PNP in CaEGTA at 1°C, then washed in rigor solution, the (1,0) intensity was lower than the original rigor value, and the (1,1) was higher. This was likely due to the high concentration of the nucleotide. For this reason, each muscle fiber was used only for one set of experiments.

Two-dimensional x-ray diffraction patterns

Two-dimensional patterns from skinned muscle bundles were taken as described in Xu et al. (1997). Experiments were carried out at 1°C, 170 mM ionic strength using Beamline X13 of the European Molecular Biology Laboratory (EMBL) at the Deutsches Elektronen Synchrotron (DESY) in Hamburg Germany. The same muscle bundle that was used for rigor, (MgATP-relaxed), and MgAMP-PNP relaxed patterns. The solution in the chamber used for holding the muscle bundle was continuously mixed by a syringe pump at ~ 1 ml/s in order to keep the solution and temperature homogeneous. The chamber was continuously moved up and down at 4 mm/s to minimize damage caused by the strong x-ray radiation. Patterns were collected at the synchrotron for 4 min under each condition tested. Two-dimensional patterns were recorded on Fuji imaging plates and scanned using a BAS2000 scanner (Fuji, Japan). The data were later transferred to a Silicon Graphics workstation for analysis. The raw data was rotated, translated, and then folded to average three usable quadrants from one exposure (the fourth quadrant is unusable because of the beam stop).

Integrated intensity profiles were obtained by taking 0.001 \AA^{-1} horizontal slices or 0.014 \AA^{-1} vertical slices. The intensities were determined using the PCA program (Nucleus, Oak Ridge, TN).

Equatorial x-ray diffraction patterns taken under other conditions

X-ray diffraction experiments were performed on single skinned fibers at ionic strengths of 75, 120, 170, and 220 mM (1°C). This was done to further characterize the cross-bridges. In another set of experiments, the temperature was gradually increased from 1° to 30°C in the presence of 10 mM MgAMP-PNP in EGTA solution. The equatorial diffraction patterns were recorded at 5°C intervals. At low temperature, the pattern was almost completely relaxed. At 30°C the equatorial pattern was rigor-like. To determine if the rigor-like pattern at elevated temperature was due to the cross-bridges being unsaturated with nucleotide, we added extra nucleotide until a concentration of 30 mM MgAMP-PNP was reached.

RESULTS

Determination of the concentration of MgAMP-PNP required to saturate cross-bridges in muscle fibers

The equatorial intensity ratio I_{11}/I_{10} was used as a measure of the relative fraction of cross-bridges that are attached or detached. In order to determine how much MgAMP-PNP was necessary to saturate the cross-bridges, the equatorial intensity ratio I_{11}/I_{10} was followed as a function of increasing MgAMP-PNP concentration as shown in Fig. 1. The curves are normalized with respect to rigor in EGTA solution (the ratio is set to one). The normalized ratio decreases as the cross-bridges detach and is ~ 0.3 for a fully MgATP-relaxed fiber at $\mu = 170$ mM (Fig. 1). Saturation has been reached by 20 mM MgAMP-PNP since the equatorial patterns for muscle fibers relaxed with MgATP and with 20

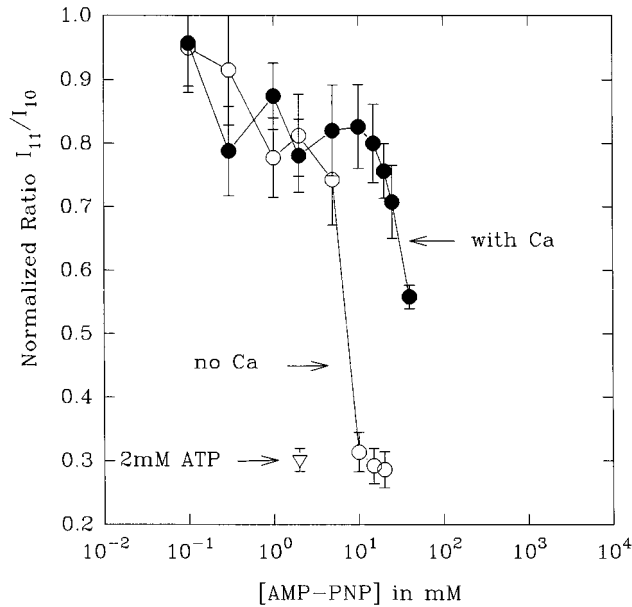


FIGURE 1 The equatorial intensity ratio $I_{(1,1)}/I_{(1,0)}$ used as a measure of the fraction of cross-bridges attached to actin as a function of increasing MgAMP-PNP concentration. Single rabbit psoas muscle fibers were studied in CaEGTA (●), in EGTA solution (○), and in 2mM MgATP with no AMP-PNP (▽). Temperature, 1°C. Ionic strength, 170 mM. Sarcomere length, 2.3–2.4 μm . The rigor data point in EGTA solution was set to 1.0 (the actual ratio was ~ 3.0). Other data points were normalized to this value.

mM MgAMP-PNP in EGTA are very similar, and the intensity ratio levels off at approximately this concentration.

In the CaEGTA solution, the intensity ratio decreased with increasing concentration of MgAMP-PNP, but did not level off even at 40 mM MgAMP-PNP (Fig. 1). The high I_{11}/I_{10} ratio was not due to activation brought about by contaminant ATP since the striation pattern under the optical light microscope remained constant, the laser diffraction pattern remained ordered, and if the muscle had been activated, the fiber would be expected to break as a result of the strain. Thus

saturation had not been reached even by 40 mM nucleotide in Ca^{2+} .

Binding of S1-MgAMP-PNP to reconstituted thin filaments in solution

The association constant for S1-MgAMP-PNP binding to the actin-troponin-tropomyosin complex was found to be in the range of $2.0 - 6.7 \times 10^3 \text{ M}^{-1}$ with a median value of $4.0 \times 10^3 \text{ M}^{-1}$ at 25°C, 75 mM ionic strength, 20 mM MgAMP-PNP in EGTA solution as shown in Fig. 2 A. Fig. 2 B shows the ionic strength dependence of the binding constant between $\mu = 75$ and 220 mM. The binding constants are consistent with those of S1-ATP at the low range of ionic strengths when extrapolated (Fig. 2 B; Chalovich et al., 1983; Highsmith and Murphy, 1992; Chalovich, Sen, and Resetar, unpublished result). For comparison, at a lower concentration of MgAMP-PNP (2 mM) at $\mu = 170$ mM the binding constant is approximately one order of magnitude higher ($\sim 5 \times 10^3 \text{ M}^{-1}$, data not shown) and is consistent with previously reported data (Greene, 1981). This observation is consistent with the fiber studies in that changes in cross-bridge properties occur when the concentration of MgAMP-PNP is raised above those commonly used.

X-ray diffraction patterns under saturating concentrations of MgAMP-PNP, characteristics similar to those under saturating concentrations of MgATP

Two-dimensional x-ray diffraction patterns were taken of MgATP- and MgAMP-PNP-relaxed muscle bundles. Fig. 3 compares the two-dimensional x-ray diffraction patterns of a muscle bundle in rigor (A), the same bundle relaxed with MgATP (B), and the same bundle relaxed with 25 mM MgAMP-PNP in EGTA solution (C). The actin-based layer lines, including those at 365, 243, 70, and 59 \AA , are very prominent in the rigor pattern. There is noticeable sampling

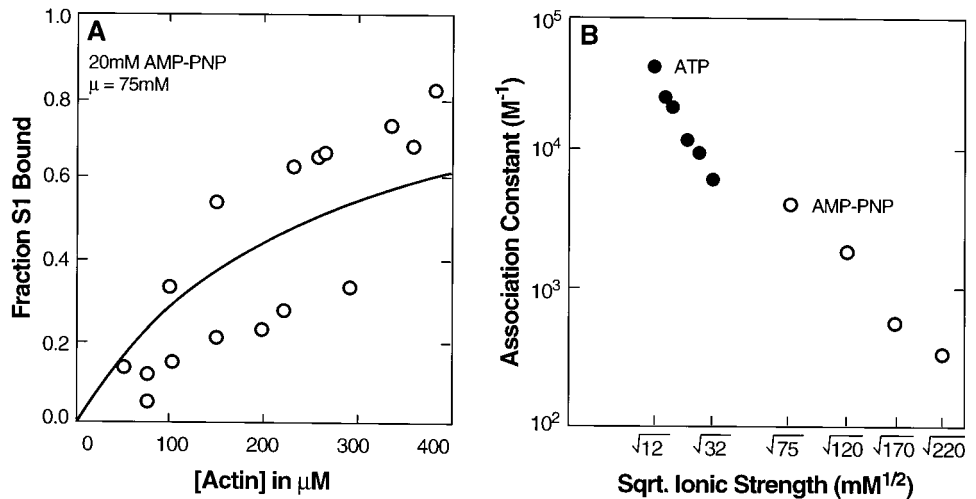


FIGURE 2 (A) The fraction of S1-MgAMP-PNP bound as a function of actin concentration used to determine the association constant at 75 mM ionic strength. (B) Association constants of S1-MgAMP-PNP and S1-MgATP for the actin-troponin-tropomyosin complex as a function of ionic strength. Constants were determined at 25°C. The association constants for MgATP are based on a variety of sources: Chalovich et al., 1983; Highsmith and Murphy, 1992; Chalovich, Sen, and Resetar, unpublished results.

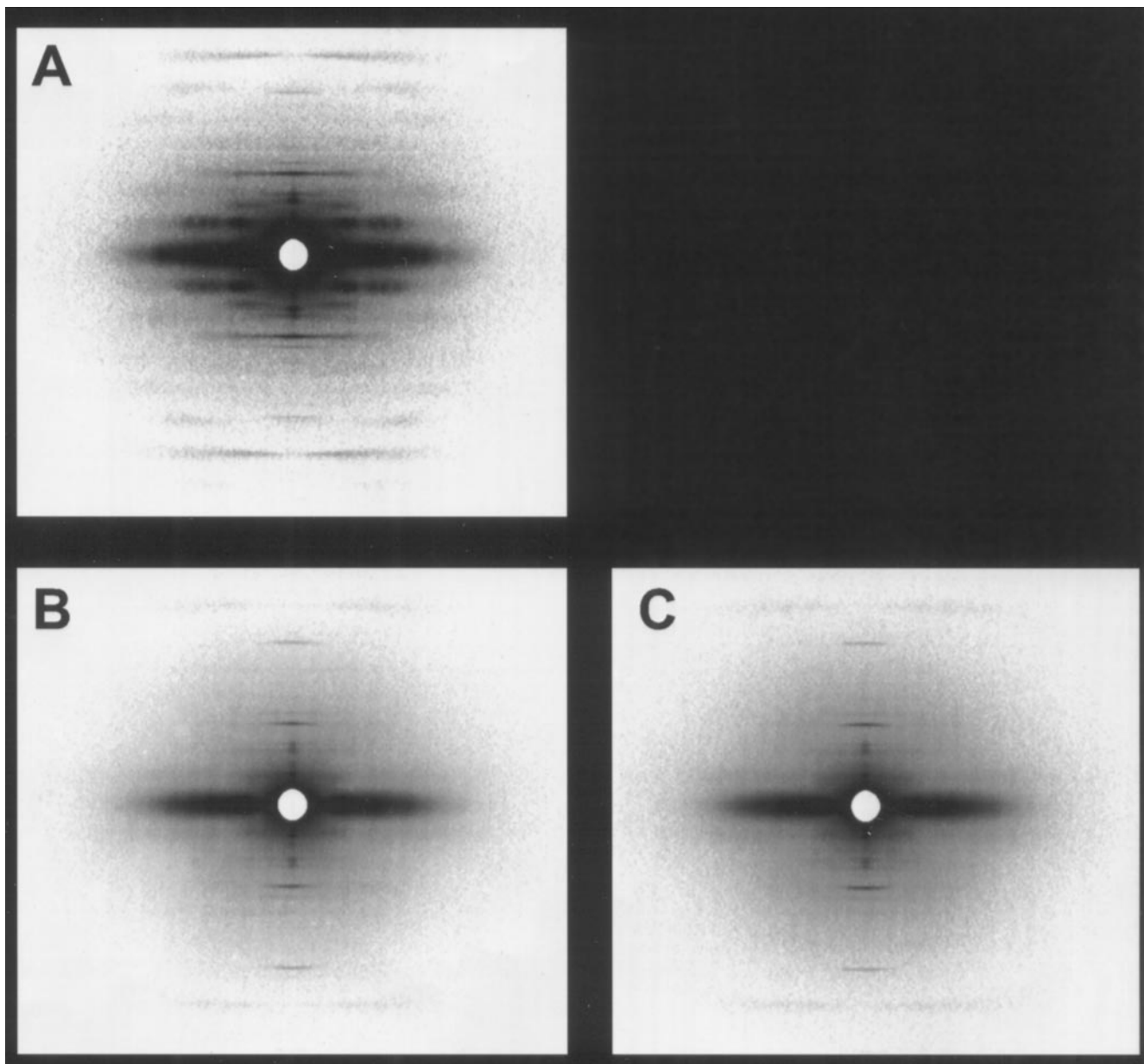


FIGURE 3 Two-dimensional x-ray diffraction patterns of one muscle bundle in rigor (*A*), in 10 mM MgATP (*B*), and in 25 mM MgAMP-PNP solution (*C*). The fiber bundle used was assembled from approximated 30 single skinned fibers. The ionic strength was 170 mM, temperature = 1°C. For the MgATP-relaxed pattern, two exposures were averaged together, one taken before treatment with 25 mM MgAMP-PNP and one taken after. The patterns were recorded using the X13 beamline at EMBL Outstation (DESY, Hamburg, Germany) with image plates and scanned by BSA2000 (Fuji, Japan).

of the decorated first actin layer line, and the 59 Å reflection is more intense and closer to the meridian than in the relaxed patterns. The two relaxed patterns shown in Fig. 3, *B* and *C*, appear to be very similar. The actin-based layer lines are mostly absent and replaced by myosin-based layer line at 430 Å which is particularly weak at this temperature (Xu et al., 1997). A higher concentration of MgAMP-PNP (25 versus 20 mM) was necessary to fully relax the muscle when a bundle was used compared with the single fiber experiments. This probably was due to a concentration gradient where the concentration is lower in the core of the bundle.

For a more detailed comparison, profiles along off-meridional vertical cuts are shown in Fig. 4, *a* and *b*, and cuts

along the meridian are shown in Fig. 5, *a* and *b*. The off-meridional layer lines (Fig. 4, *a* and *b*) are very similar; the absence of the actin layer lines and the weak myosin layer lines as reported in an earlier study (Xu et al., 1997). The meridional profiles shown in Fig. 5 are comparable for MgATP and MgAMP-PNP relaxed muscle, but there are some differences. The intensity of the group of reflections at 215 Å, which arises from the perturbation of the myosin helix (Yagi et al., 1981; Malinchik and Lednev, 1992), is ~42% lower in the MgAMP-PNP patterns. In addition, the meridional 145 Å myosin-based reflection is ~34% higher in the MgAMP-PNP case. After exposure to MgAMP-PNP, the group of reflections at 215 and 72 Å reflections recov-

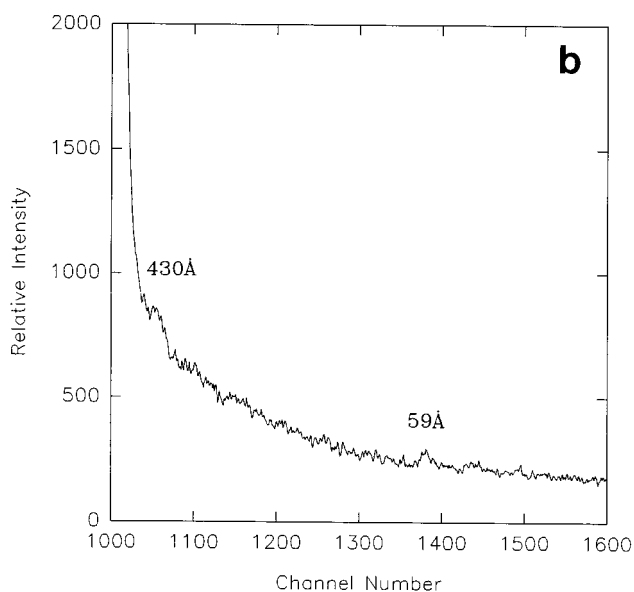
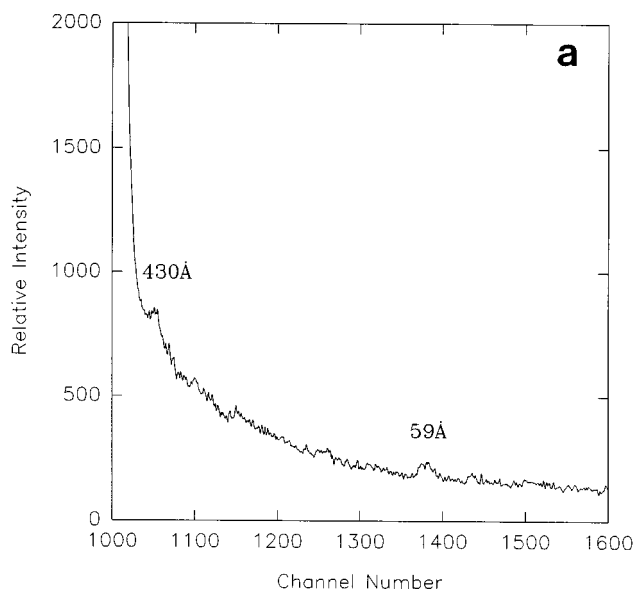


FIGURE 4 Profiles of off-meridional vertical slices through the two-dimensional x-ray diffraction patterns shown in Fig. 3. (a) 10 mM MgATP. (b) 25 mM MgAMP-PNP. Myosin layer line at 430 Å and actin layer line at 59 Å are indicated.

ered completely, but the 145 Å reflection increased by ~38%. This shows that while the changes observed upon MgAMP-PNP binding are essentially reversible, there is still a structural component that does not recover upon removal of this nucleotide.

Dependence of the equatorial intensity ratio I_{11}/I_{10} on the ionic strength

Nonactivating cross-bridges are sensitive to ionic strength. Experiments were performed in which the equatorial intensity ratio was measured as a function of ionic strength to

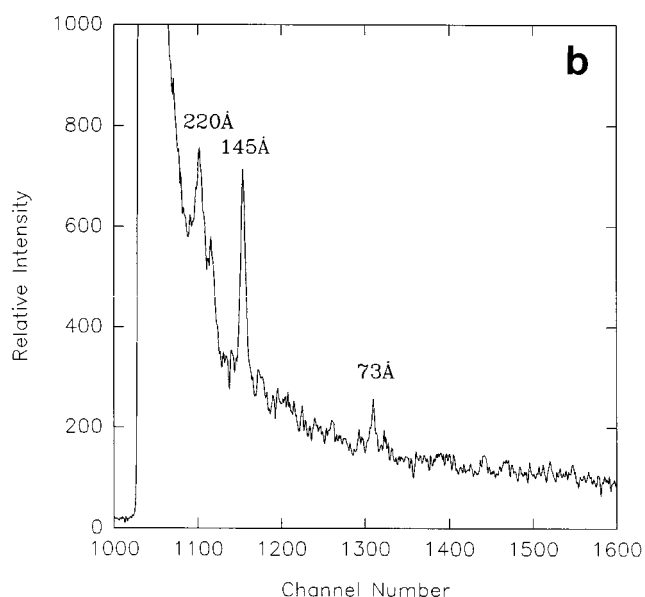
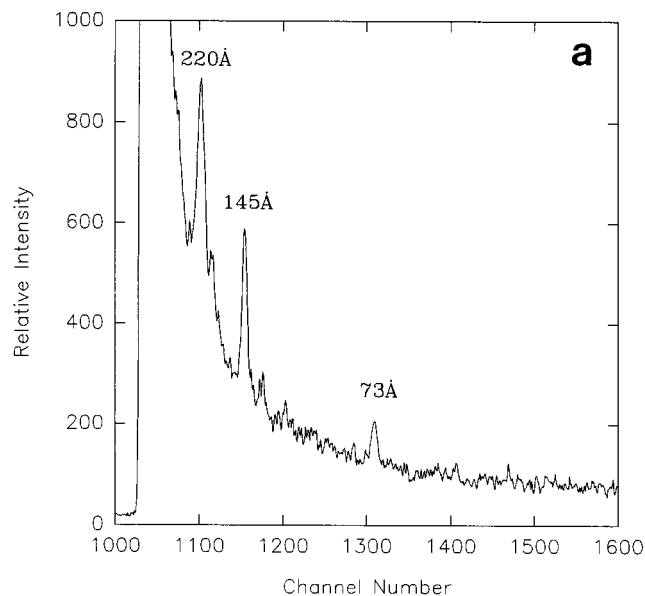


FIGURE 5 Meridional profiles of a MgATP-relaxed bundle (a) and the same bundle relaxed in MgAMP-PNP (b). Thick filament related meridional reflections at 220, 145, and 73 Å are indicated.

determine if the MgAMP-PNP cross-bridges would behave similarly to other nonactivating cross-bridges. The ionic strength dependence of the I_{11}/I_{10} of fully relaxed fibers in 10 mM MgATP and 20 mM MgAMP-PNP is shown in Fig. 6 A. A concentration of 20 mM MgATP to match the concentration of MgAMP-PNP was not possible since at high concentrations of MgATP, low temperature, and high ionic strength, the x-ray diffraction pattern becomes disordered. As is the case under relaxing MgATP conditions, at 20 mM MgAMP-PNP the equatorial intensity ratio decreases as the ionic strength increases showing a trend consistent with nonactivating cross-bridges. The lowest ionic strength we could attain was 75 mM due to the high concentration of MgAMP-PNP.

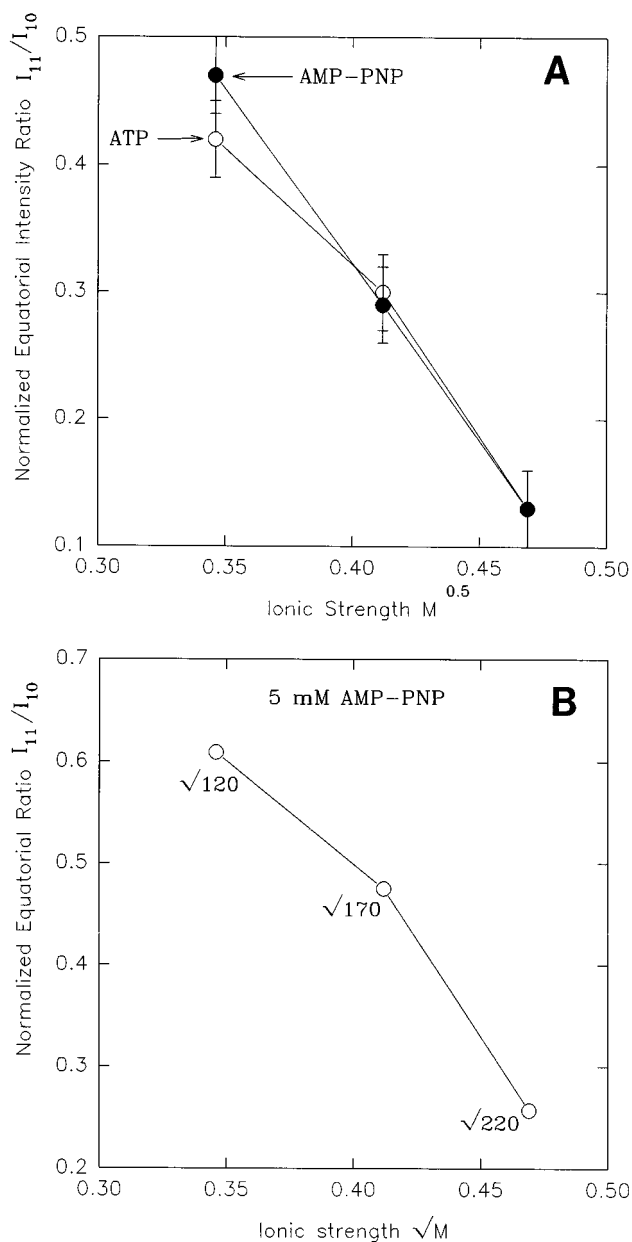


FIGURE 6 (A) Ionic strength dependence of the I_{11}/I_{10} intensity ratio for fibers in saturating MgATP (10 mM) (○) and MgAMP-PNP (20 mM) (●) showing the similarity in behavior in the two nucleotides. (B) Ionic strength dependence of the I_{11}/I_{10} intensity ratio of a fiber at 5 mM MgAMP-PNP. At high ionic strengths, even at 5 mM of AMP-PNP, the fiber is close to be relaxed, suggesting that at lower ionic strengths more nucleotide is required to reach saturation. The temperature was maintained at 1°C for all experiments, and the patterns were normalized to rigor condition, which is equal to 1.0.

The effect of ionic strength on the saturation of cross-bridges with nucleotide was also investigated as shown in Fig. 6 B. At 5 mM MgAMP-PNP and low ionic strength the pattern is rigor-like. As the ionic strength increases, the fiber approaches the relaxed value. Therefore, at low ionic strength it requires more MgAMP-PNP to saturate the rigor cross-bridges.

Effect of temperature on saturation

Fig. 7 shows the temperature dependence of the intensity ratio. At 1°C the pattern taken in 10 mM MgAMP-PNP EGTA solution is almost fully relaxed. At 30°C and the same nucleotide concentration the same muscle fiber is in rigor. To show that raising temperature affects the saturation level, MgAMP-PNP was added gradually at this elevated temperature until a concentration of 30 mM was reached. Only at this concentration the equatorial intensity ratio I_{11}/I_{10} appeared similar to the ratio obtained at 10 mM MgATP. However, the pattern with the high nucleotide concentration and high temperature was very poor, and quantitative determination of the intensities was not possible.

Investigation of contamination in MgAMP-PNP

There was one major contaminant found in the MgAMP-PNP by HPLC (first peak in Fig. 8 A). This was determined to be the degradation product AMP-PN (see Materials and Methods). Preliminary mechanical data (Frisbie and Kraft, unpublished data) show that the stiffness was higher when the AMP-PN contamination was ~25%. Only freshly opened bottles of MgAMP-PNP were used in order to minimize this component to ~5%.

As shown in Fig. 8 B, there was no ATP detected by HPLC even when the column was overloaded with 10 μ l of 20 mM AMP-PNP. Under the same conditions, it was possible to detect as little as 10 μ l of 0.5 μ M of ATP by HPLC (Fig. 9, A and B). As a control, HPLC traces were taken both before and after x-ray diffraction experiments to make sure no ATP was detectable. No evidence of ATP caused by insufficient washing of the fiber and x-ray chamber was found.

At 20 mM MgAMP-PNP, could the relaxation observed in a muscle fiber be caused by 0.5 μ M MgATP contamination as this is the limit of our detectability? This question can be answered by comparing the concentrations of MgATP-relaxed (MT) and MgAMP-PNP-relaxed (MN) cross-bridges using the program Mathematica. Table 1 shows the results of the calculations. Values are given using actin concentrations of 300 and 50 μ M since this is the range used to determine the association constant of S1-MgAMP-PNP binding to the actin-troponin-tropomyosin complex measured *in vitro*. Also shown are the values obtained using an effective actin concentration of 0.5 mM and a myosin concentration of 5 μ M for muscle fibers (see Discussion). At the concentrations of actin used in our solution studies, the concentration of MgATP-relaxed cross-bridges is approximately one order of a magnitude less than the MgAMP-PNP-relaxed fibers. For the fiber-specific actin and myosin concentrations, the MgATP-relaxed cross-bridges are also approximately one order of magnitude less than the MgAMP-PNP-relaxed cross-bridges. Even at concentrations of 1 μ M MgATP, twice that which it is possible to observe by HPLC (although we see

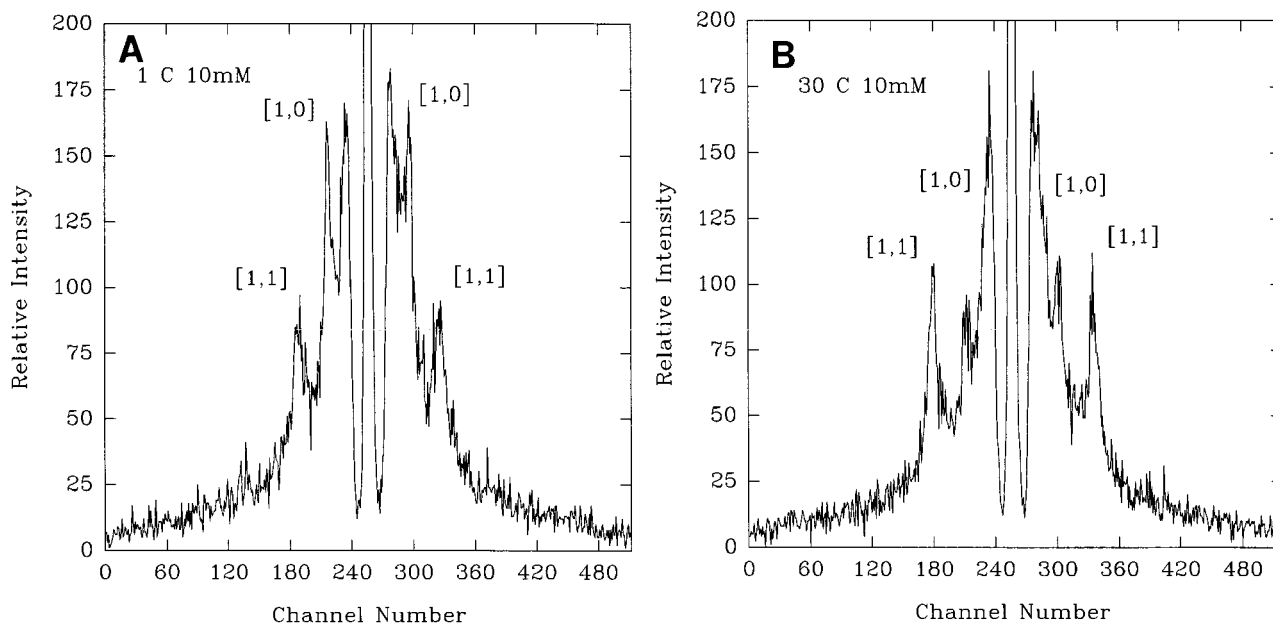


FIGURE 7 Temperature dependence of the x-ray equatorial intensity ratio I_{11}/I_{10} . (A) At 1°C, the pattern at 10 mM MgAMP-PNP is relaxed. (B) At 30°C, the same muscle fiber is in rigor. When the concentration of MgAMP-PNP was increased to 30 mM, I_{11} decreased greatly, rendering the diffraction pattern relaxed-like. However, the quality of the pattern was too poor for quantitative analysis.

none), the concentration of relaxed cross-bridges caused by MgATP would be insufficient to explain the present findings.

DISCUSSION

Perhaps the most important implication of the present study is that the cross-bridge state formed in the presence of saturating MgAMP-PNP is not, as often assumed, a strong binding, activating state. While low concentrations of MgAMP-PNP do result in strong binding behavior, it is likely that this is because of incomplete saturation of myosin with MgAMP-PNP. Thus, experiments using MgAMP-PNP appear straightforward but may actually contain hidden complexities because of the distribution of states. This situation is far worse if low concentrations of MgAMP-PNP are used in studies of regulation, because binding of S1 to MgAMP-PNP, similar to other nucleotides recently studied (Frisbie et al., 1997a), is Ca^{2+} -dependent. Thus, much higher concentrations of MgAMP-PNP are required to elicit a maximum effect in the presence of Ca^{2+} .

Determination of nucleotide concentration required for saturation

It has been shown that the equatorial intensity ratio may change as a result of a change in the number of cross-bridges attached and/or a conformational change in the attached myosin (Lynn, 1978; Yu, 1989; Malinchik and Yu, 1995). Concerns may be raised whether the intensity ratio I_{11}/I_{10} should be used as an indicator for saturation of nucleotide. The key point here is that regardless of the mechanism, it is the leveling off in the change of I_{11}/I_{10} as

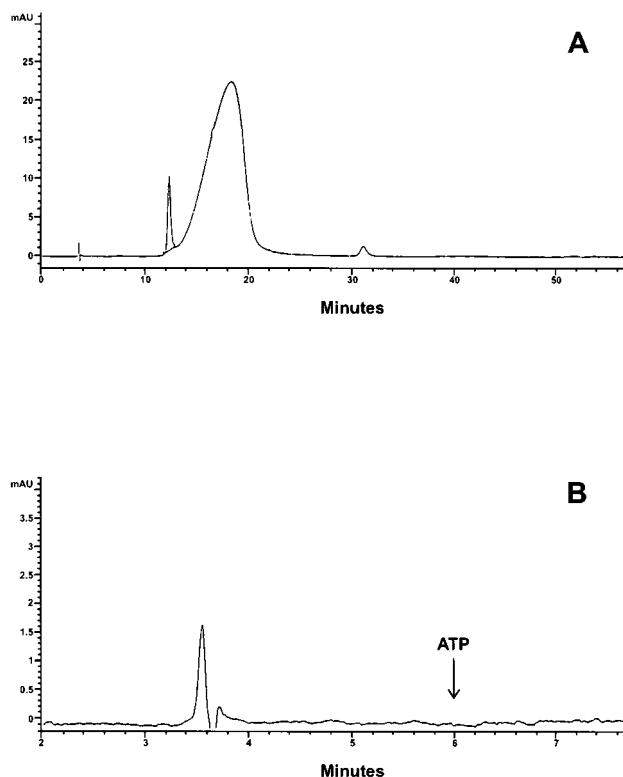


FIGURE 8 (A) HPLC of AMP-PNP and ATP showing the limit of possible ATP contamination in the AMP-PNP used. No ATP was detected in the AMP-PNP used in the x ray and solution experiments. (B) Expanded region where ATP is expected.

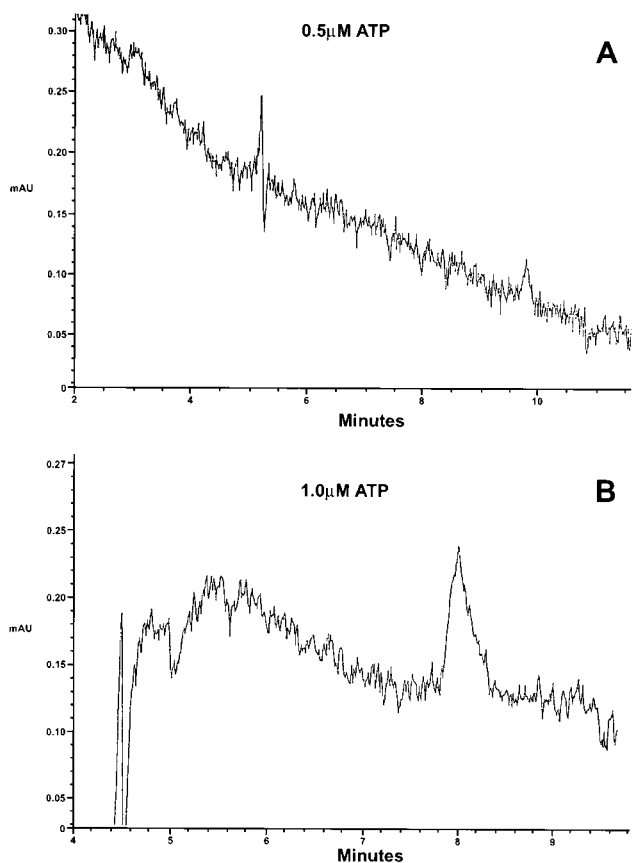


FIGURE 9 HPLC traces of 0.5 μM (A) and 1 μM (B) ATP used to determine the limit of detectability of this nucleotide. All samples were run under the same conditions as in Fig. 8.

a function of the nucleotide concentration (Fig. 1) that led to our conclusion. Furthermore, our findings of the low affinity of myosin for MgAMP-PNP in skeletal muscle are in close agreement with earlier results of Fajer et al. (1988) and of Biosca et al. (1988). However, based on equatorial x-ray diffraction alone a simple conformational change leading to a relaxed intensity ratio cannot be completely ruled out.

Unlike EGTA solution, in CaEGTA solution saturation had not been reached even at a nucleotide concentration of 40 mM. The ratio also never reached a constant value, and it was not possible to use a higher concentration of MgAMP-PNP while retaining a 170 mM ionic strength. This pronounced calcium sensitivity to nucleotide saturation supports our similar finding with MgGTP in rabbit psoas fibers (Frisbie et al., 1997a).

It might be argued that the high concentrations of MgAMP-PNP used in the present study may have caused some structural instability in myosin that could lead to an apparent relaxation of the muscle fibers. However, the decrease in the intensity ratio I_{11}/I_{10} was monotonic and gradual (Fig. 1). The equatorial diffraction patterns remained sharp at 20 mM MgAMP-PNP (in EGTA), indicating structural stability. Furthermore, the ATP-like behavior under MgAMP-PNP is essentially reversible (Figs. 1 and 3).

Therefore, the high concentration required for saturation is most likely due to low affinity of myosin for MgAMP-PNP in skeletal muscle.

Cross-bridges are in nonactivating cross-bridge states in the presence of saturating concentrations of MgAMP-PNP

Several lines of evidence point to our conclusion that cross-bridges are in weak, nonactivating states in the presence of MgAMP-PNP: 1) the x-ray diffraction patterns of MgAMP-PNP-relaxed fibers closely match those in MgATP (albeit with some differences on the meridian); 2) the association constant of S1-MgAMP-PNP binding to reconstituted thin filaments is comparable with that of S1-MgATP; 3) in the presence of a saturating concentration of MgAMP-PNP, the thin filament is not activated; and 4) the ionic strength dependence of the equatorial diffraction pattern is similar for MgAMP-PNP and MgATP.

Our results are consistent with reports that the atomic structure of S1-MgAMP-PNP is similar to S1-MgATP γ S (Gulick et al., 1997), a nonactivating (weak) binding analog (Kraft et al., 1992). An overlay of the x-ray crystallographic data indicates that both the actin binding regions and the nucleotide binding pockets correspond very closely for these two structures. The data are essentially superimposable with the x-ray data on the S1dC structure with the weak binding analog MgADP-BeF_x (beryllium fluoride) as well (Fisher et al., 1995). However, the side chain oxygen of Asn²³³ of the S1dC.MgAMP-PNP is rotated to interact with the bridging nitrogen. This disrupts the water structure surrounding the ribose hydroxyl group. It is possible that this results in a reduced affinity of S1 for the nucleotide (Gulick et al., 1997).

Other recent experiments are also consistent with MgAMP-PNP being a weak-binding analog under saturating conditions. Skinned fibers saturated with MgAMP-PNP behave mechanistically like fibers completely relaxed with MgATP (Heizmann et al., 1997). Electron paramagnetic resonance experiments by Arata (1990) indicate that the myosin heads with bound MgAMP-PNP are either weakly bound or detached from the thin filament, and under low ionic strength conditions the cross-bridges appear rigor-like suggesting nonsaturation.

Effective actin concentration

The effective actin concentration (A_{eff}) is the concentration of actin required in solution to reach the same fraction of actin bound to myosin as in a fiber. It is defined as K_b/K_{solution} as shown below:

In muscle fibers, the equilibrium constant K_b is defined as:

$$K_b = \frac{\text{(fraction of attached cross-bridges)}}{\text{(fraction of detached cross-bridges)}}$$

In solution, the equilibrium constant is:

$$K_{\text{solution}} = \frac{(\text{fraction of attached S1})}{(A)_{\text{eff}}(\text{fraction of detached S1})}$$

Previously, the effective actin concentration was estimated to be in the range of 3 mM (Brenner et al., 1986) using 4 mM pyrophosphate (MgPPi) in solution and in skinned fibers. An assumption used to calculate the effective actin concentration was that the concentration of MgPPi required for saturation was the same in solution as in fibers, and that saturation had been reached at 4 mM MgPPi. Using the present results under saturating MgAMP-PNP conditions, the effective actin concentration is again estimated. Using a similar algorithm of comparing the binding data obtained in solution and in fibers under various ionic strengths (Figs. 2 and 6A), the effective actin concentration is estimated to be ~0.5 mM, somewhat lower than the previous estimate.

Summary

Our major conclusion is that in the presence of a saturating concentration of MgAMP-PNP cross-bridges are in weak-binding, nonactivating states. Consequently, it would be unsuitable to use the cross-bridges in the presence of this nucleotide to study the activating state. During the course of the present study, several elements were found to be critical in saturating the cross-bridges with MgAMP-PNP: low temperature, high ionic strength, and purity of the nucleotide (little or no degradation products and no detectable ATP). Many of the previous studies that resulted in a mixture of cross-bridge states, an intermediate state, or strong activating states could be due to lack of cross-bridge saturation with nucleotide that could occur when one or more of these conditions are not met. There are some indications that the level at which saturation is reached is species specific and may be different in different muscle types (Reedy et al., 1983, 1987; Biosca et al., 1990). This could be a source of confusion regarding the state of actomyosin cross-bridges in the presence of MgAMP-PNP.

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