Modulation of Cross-Bridge Affinity for MgGTP by Ca^{2+} in Skinned Fibers of Rabbit Psoas Muscle

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ABSTRACT Previously we reported that saturation of cross-bridges with MgATPγS in skinned muscle fibers was calcium sensitive. In the present study we investigate whether this observation can be generalized to other nucleotides by studying saturation of cross-bridges with MgGTP. In solution, myosin-subfragment 1 (S1) in the presence of 10 mM MgGTP was found to bind to actin with low affinity, similar to that in the presence of MgATP and MgATPγS. In EGTA buffer, the equatorial x-ray diffraction intensity ratio I_{11}/I_{10} recorded in single skinned fibers decreased upon increasing MgGTP concentration from 0 to 10 mM (1°C and 170 mM ionic strength). The I_{11}/I_{10} ratio leveled off at 10 mM MgGTP, indicating full saturation of cross-bridges with the nucleotide. Under these conditions, the value of I_{11}/I_{10} is indistinguishable from that obtained in the presence of saturating [MgATP]. In CaEGTA buffer, however, the decrease in I_{11}/I_{10} occurs over a wider range of concentrations, and there is no indication of I_{11}/I_{10} leveling off at 10 mM MgGTP, suggesting that full saturation is not reached. The Ca^{2+} dependence of GTP binding appears to be a direct consequence of the differences in the affinities of the strongly bound cross-bridges to actin versus weakly bound cross-bridges to actin. A biochemical scheme that could qualitatively explain the titration behavior of ATPγS and GTP is presented.

INTRODUCTION

The two major components of skeletal muscle are the thick and the thin filaments. Thick filaments consist mainly of myosin, whereas thin filaments consist of actin, the troponin complex, and tropomyosin. Cross-bridges projecting from the thick filaments bind to the thin filaments in an ATP-dependent cyclic manner to produce force. The interaction between the myosin cross-bridges and actin can be broadly characterized as weak/nonactivating states or strong/activating states (Eisenberg and Hill, 1985; Brenner, 1990; Chalovich, 1992). Weakly bound cross-bridges are in pre-force-generating states, which are required for force development later in the contraction cycle (Brenner et al., 1982, 1991; Kraft et al., 1995a). They are characterized by their inability to activate the contractile system (Chalovich and Eisenberg, 1982). They also have a low affinity for actin that is ionic strength dependent. Strongly binding cross-bridge states are the major force-generating states (Eisenberg and Hill, 1985) and have a greater affinity for actin than the weakly binding cross-bridge states when measured under identical experimental conditions.

It is well accepted that the binding of calcium to troponin C changes the thin filament from an inactive to an active form that can increase the rate of ATP hydrolysis by myosin. However, details of this activation of ATP hydrolysis continue to be a matter of discussion. In particular, although there is agreement that Ca^{2+} affects the strength of binding of strong/activating states to actin, there is disagreement as to the extent to which Ca^{2+} can control the affinity of weak/nonactivating states for actin. It was reported previously that cross-bridges in the presence of several ATP analogs such as ATPγS and PrNANTP (3-[(4-azido-2-nitrophenyl)amino]propyl triphosphate) formed weakly binding cross-bridge states. Such cross-bridges were found to bind to actin in a highly Ca^{2+}-dependent manner (Dantzig et al., 1988; Pate et al., 1991), as if Ca^{2+} controlled the affinity of myosin to actin. In a subsequent study of the state formed in the presence of ATPγS, the affinity of cross-bridges for actin, however, was found not to be significantly affected by calcium activation; only the kinetics for association and dissociation to and from actin were slowed (Kraft et al., 1992). Unexpectedly, the affinity of ATPγS for cross-bridges was found to be highly Ca^{2+} sensitive. Full saturation of the cross-bridges with ATPγS was reached at approximately 50 μM in EGTA-containing solution, and at approximately 15 mM in CaEGTA solution, where the thin filament was activated. The Ca^{2+} effects on cross-bridges weakly attached to actin described in earlier studies (Dantzig et al., 1988; Pate et al., 1991) were shown to be due to incomplete saturation of myosin with ATPγS at high $[Ca^{2+}]$.

To investigate whether the Ca^{2+} dependence of saturation of weakly attached cross-bridges with ATPγS is a general property of nucleotides, we have chosen to investigate the calcium effect using MgGTP, a slowly hydrolyzable nucleotide. Nucleotides with slow turnover rates were studied instead of ATP, since rapid hydrolysis of ATP leads to a mixture of cross-bridge states, which complicates data interpretation.

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We found that the behavior of binding of MgGTP to actomyosin cross-bridges is very similar to that of MgATPγS; i.e., it is an ATP analog and its affinity to cross-bridges is Ca^{2+} dependent.

Preliminary results were previously reported (Frisbie et al., 1994).

MATERIALS AND METHODS

Fiber preparation

Single skinned rabbit psoas muscle fibers used in x-ray diffraction studies were prepared and mounted as previously described (Brenner et al., 1982; Yu and Brenner, 1989; Kraft et al., 1995). 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 that 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 a fiber to the carbon tips in the specimen chamber.

Solutions

The solution used to chemically skin the fresh rabbit psoas muscle fibers contained 0.5% Triton X-100, 5 mM KH_2PO_4, 3 mM Mg acetate, 5 mM EGTA, 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 (E64; Calbiochem-Novabiochem Corp., La Jolla, CA), 0.01 mM pepstatin, and 1 mM 4-(2-aminoethyl)-benzenesulfonylflouride (AEBSF). The MgGTP solutions used were composed of the following: 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 Ap_5A (p^1,p^5-di(adenosine-5-pentaphosphate) from Sigma, St. Louis, MO). Hexokinase was added to convert any contaminating ATP to ADP that might be present from the skinning solution. For 0–1 mM GTP, 1 unit/ml of hexokinase and 50 mM glucose were used; for 5–10 mM, 3 units/ml and 60 mM, respectively, were used. Ap_5A was added to prevent myokinase from synthesizing ATP from any contaminant ADP. MgGTP was added up to 10 mM in a stepwise fashion. The rigor solutions were identical to the MgGTP 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. The ionic strength of all other solutions was maintained at 170 mM by adding appropriate amounts of potassium propionate.

GTP was either purchased from Boehringer Mannheim Biochemicals (Indianopolis, IN) and purified as described previously for ATPγS (Kraft et al., 1992), or purchased in ultrapure grade from Pharmacia (Uppsala, Sweden). The purity of GTP was checked by both high-performance liquid chromatography (HPLC) on a Phenomenex (Torrance, CA) Primesphere 5-μm C_{18} HC 250 × 4.6 mm HPLC column, using a Hewlett-Packard (Palo Alto, CA) 1090 System, and thin-layer chromatography. The purified GTP was found to contain approximately 4% GDP. The ultrapure grade GTP from Pharmacia contained no detectable GDP. X-ray diffraction data shown in Fig. 2 were obtained with both purified GTP and ultrapure grade GTP. For binding experiments, the ultrapure grade was used.

Measurement of the association constant of actin-troponin-tropomyosin to S1-GTP

The association constants between the reconstituted thin filament and S1-GTP under EGTA and CaEGTA conditions were obtained at 25°C as described previously (Chalovich and Eisenberg, 1982).

Measurement of GTPase rates

MgGTPase and MgATPase measurements were done in parallel using the same myofibril preparation and the same conditions (4°C and μ = 170 mM) and solutions (except the nucleotides), without a creatine phosphokinase backup system. Approximately 50 μl of homogenized skinned rabbit psoas muscle (myofibrils) was added to 250 μl of a solution containing 10 mM ATP or GTP with EGTA or CaEGTA. Changes in the concentration of either ATP and ADP or GTP and GDP were followed by using the HPLC Phenomenex column described above. Nucleotides were eluted using an isocratic 5 mM sodium phosphate solution (pH 5.2). Reactions were stopped by quenching of 10-μl aliquots with 10 μl of 0.6 M perchloric acid. The GTPase rates were measured as a percentage of the ATPase rates measured in CaEGTA solution.

X-ray diffraction studies

The equipment and procedure for recording equatorial x-ray diffraction patterns were described previously (Brenner et al., 1984). Patterns for relaxed fibers at μ = 20 mM and 170 mM were collected first. Each fiber was then washed extensively with a rigor solution before the addition of nucleotide. During each titration (i.e., from rigor to 10 mM MgGTP) the x-ray beam was directed at a single spot on the muscle fiber. The [MgGTP] was gradually increased, with CaEGTA and EGTA solutions being alternated such that the data for the two titration curves were taken in series. Ordinarily exposure times would be 500 s for each data point at different places along the fiber. The present technique of exposing the same spot along the fiber reduced uncertainty in the data by reduction of disorder and inhomogeneity effects present in the muscle preparation. As a result, it was possible to take exposures of 100 s for each data point. Muscle damage was minimized by keeping the total x-ray exposure time for each spot on the muscle below 2700 s.

The reversibility of MgGTP binding to cross-bridges was checked after the fiber had been incubated with 10 mM MgGTP. No permanent effect was observed on muscle fibers. The fibers could be relaxed with ATP and could form rigor cross-bridges, indistinguishable from untreated fibers as determined by x-ray diffraction.

The integrated equatorial intensities of the I_{11} and I_{10} reflections were determined by using the MLAB curve-fitting program from Civilized Software (Bethesda, MD), as described by Yu and Brenner (1989).

RESULTS

Association constants for S1-GTP binding to the thin filament in solution

In the presence of GTP, S1 binds to actin with an affinity that is characteristic of weakly binding states. The fraction of S1-GTP bound as a function of the free actin concentration at 70 mM ionic strength is shown in Fig. 1. The association constants were estimated by hyperbolic fits to be $4 \times 10^3\ M^{-1}$ in CaEGTA and EGTA solutions. (Experimental error is approximately 10–20%.) From preliminary experimental data as well as an extrapolation of the 70 mM ionic strength data, we estimate the association constant to be approximately $1 \times 10^3\ M^{-1}$ at μ = 170 mM. This value is similar to the association constant for S1-ATP extrapolated from lower ionic strength data in the range of μ = 10–50 mM reported earlier (Chalovich and Eisenberg, 1982). The data show that the binding of S1-GTP to actin is little affected by the state of the thin filament (in the presence or absence of Ca^{2+}), a typical property of weakly binding states.

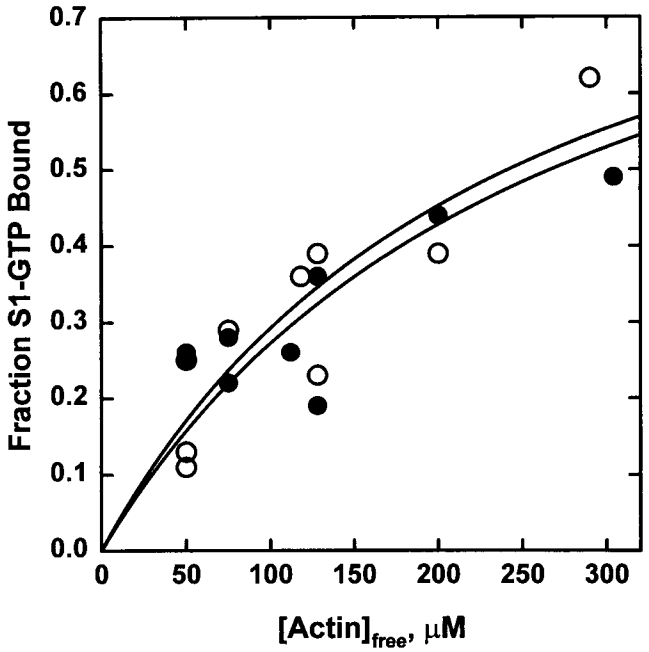

FIGURE 1 Binding of S1-GTP to actin-troponin-tropomyosin in EGTA (○) and CaEGTA solutions (●) at μ = 70 mM and 25°C. Hyperbolic fits to the data taken at μ = 70 mM yielded the association constants to be 4 × 10^3 M^{-1} in CaEGTA and EGTA solutions. Preliminary experimental data as well as an extrapolation of the 70 mM ionic strength data estimate the association constant to be approximately 1 × 10^3 M^{-1} at μ = 170 mM.

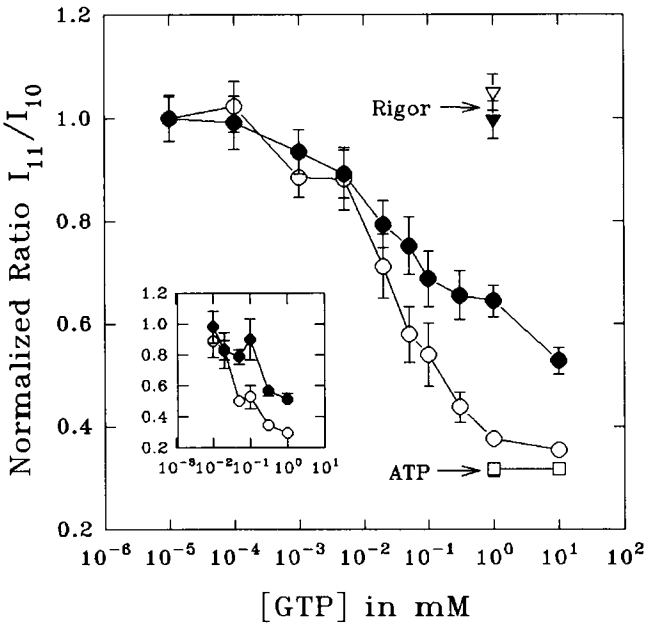

FIGURE 2 The equatorial intensity ratio $I_{1,1}/I_{1,0}$ used as a measure of fraction of cross-bridges attached to actin as a function of increasing GTP concentration. Single rabbit psoas muscle fibers were studied in CaEGTA (●) and in EGTA solution (○). The data point at 0.01 μM MgGTP was set to 1.0 (the actual ratio is ~3.0). Error bars are the standard error of the mean and represent data from 12 fibers. Fibers relaxed in 1 mM and 10 mM MgATP-EGTA solution are shown as a control for the detached state (□). Rigor in CaEGTA solution (▼) and in EGTA solution (▽) are shown as a control for the attached state. Experiments were performed at 1°C. (*Inset*) In another set of experiments, ultrapure grade GTP, together with a phosphocreatine kinase backup system (840 units/ml of CPK and 66 mM creatine phosphate), was used to remove possible GDP contamination in CaEGTA (●) and EGTA (○) solutions.

Decrease in the equatorial intensity ratio I_{11}/I_{10} as a function of increasing [MgGTP]: effects of calcium

Changes in the normalized equatorial intensity ratio I_{11}/I_{10} as a function of MgGTP concentration from 12 fibers are shown in Fig. 2. The mean value of the data points at 0.01 μM MgGTP is set to 1.0 (the actual ratio is 3.0). The normalized mean values for rigor fibers in EGTA and CaEGTA are 1.0, and for relaxed fibers in 1 mM and 10 mM MgATP are both 0.33. At the beginning of a set of experiments, each muscle fiber was in rigor. MgGTP was then added stepwise from a concentration of 0.01 μM to 10 mM. Both titration curves were obtained concomitantly, alternating between CaEGTA- and EGTA-containing solutions. There was no visible change in the intensity ratio until the concentration of MgGTP exceeded 1 μM. As the concentration of MgGTP increased, there was a monotonic decrease in the intensity ratio for both EGTA and CaEGTA conditions. However, the extent of decrease was different for the two conditions; in EGTA the change was more pronounced. At 10 mM MgGTP, in EGTA, the normalized intensity ratio levels off at a value hardly distinguishable from that in MgATP (0.35 versus 0.33), whereas in CaEGTA the normalized intensity ratio was 0.53. More importantly, in CaEGTA, the value of I_{11}/I_{10} does not reach a constant value, i.e., a value that is no longer affected by further increase in [MgGTP].

Precipitation occurred at concentrations greater than 10 mM in both EGTA and CaEGTA solutions, so that x-ray experiments were not carried beyond that limit. Since GTP is known to precipitate at high concentrations, we assume that the precipitation is due to GTP. It has been our observation that when there is any precipitation, there is significant deterioration in the diffraction pattern.

GTP and ATP hydrolysis rates of myofibrils

GTPase activities were measured for myofibrils using 10 mM GTP in the presence of EGTA and CaEGTA. The GTPase activities measured under activating and nonactivating conditions are given in Table 1. The activity of GTP

TABLE 1 Relative hydrolysis rates* for ATP and GTP in myofibrils

ATPase (EGTA)	ATPase (CaEGTA)	GTPase (EGTA)	GTPase (CaEGTA)
0.17 ± 0.04	1 ± 0.10	0.14 ± 0.07	0.05 ± 0.07

*GTPase rates were obtained with the same myofibril preparation as the ATPase rates. Experimental conditions: 4°C and 170 mM ionic strength, with no phosphocreatine kinase backup system.

hydrolysis at 4°C and 170 mM ionic strength was very low, and Ca^{2+} had little effect. The results are in general agreement with those of Pate et al. (1993).

GTP is the only cause for the observed changes in equatorial intensities

Contamination of the GTP with ATP could potentially produce the observed changes in I_{11}/I_{10}. HPLC analysis was done on both GTP stock solutions before and after x-ray experiments were performed. No peak associated with ATP was detected, placing an upper limit on possible ATP contamination to 0.06% of the GTP concentration. The actual ATP contamination could be much lower in our experiments.

To further ensure that GTP is the only source for changing the equatorial intensities, the inset in Fig. 2 shows a set of x-ray experiments performed using the ultrapure grade of GTP (Pharmacia, Uppsala, Sweden) with a phosphocreatine kinase backup system (870 units/ml with 66 mM creatine phosphate) to remove any contaminating GDP. There was little difference between the two sets of x-ray experiments. Therefore there was little GDP contamination in our experiments.

DISCUSSION

The main finding of the present study is that in the skinned fibers, the state of the thin filament has a large effect on the affinity of cross-bridges for GTP. This result is similar to the result found previously with ATPγS.

The use of I_{11}/I_{10} to monitor the saturation of nucleotide binding to cross-bridges

It might be argued that x-ray diffraction may not be an ideal technique for monitoring nucleotide binding, since changes in I_{11}/I_{10} that occur with increasing nucleotide concentrations may reflect detachment of cross-bridges and/or conformational changes of attached cross-bridges (Lymn, 1978; Malinchik and Yu, 1995). However, the function of x-ray diffraction used in this study is similar to many other spectroscopic techniques, i.e., to monitor changes in a system induced by a changing parameter (e.g., nucleotide concentration). The key point leading to our interpretation is the experimental finding that in CaEGTA the titration curve did not level off at the highest possible concentration of Mg-GTP (10 mM) (Fig. 2), whereas in EGTA I_{11}/I_{10} did level off. The failure of the signal to level off is a clear indication that saturation with GTP has not been reached in CaEGTA solution. Therefore, we are led to conclude that the affinity of GTP for cross-bridges bound to actin depends on the presence or absence of Ca^{2+}.

Analysis of the GTP and ATPγS results based on a biochemical scheme

Based on the simplest kinetic scheme in which two different states of the actin filament are defined, the observed effect of Ca^{2+} on nucleotide affinity for cross-bridges is expected (Hill et al., 1981). This scheme is illustrated in Fig. 3 *a*. The actin filament is either in an inactive, turned-off form (A_I), allowing low-affinity interaction with myosin heads, or in an activated, turned-on form (A_{II}), allowing high-affinity interaction with myosin heads. (In the paper of Hill et al. (1981), the active and inactive forms of actin were termed "weak binding" and "strong binding" forms of actin, which, later and in this discussion, were replaced to avoid confusion with the weakly and strongly binding states of the myosin head (Eisenberg and Hill, 1985).)

In the absence of Ca^{2+} (all actin in the A_I form; see scheme in Fig. 3 *b*), both nucleotide-bound *and* nucleotide-free myosin heads can only bind with low affinity to actin. Because of detailed balance, nucleotide affinity for both attached and detached myosin heads is high (qualitatively shown by length of arrows). However, reducing nucleotide concentration from full saturation will result in an increase of nucleotide-free myosin heads attached to actin that will start to activate the thin filament to the active form (A_{II}). The activation occurs as the fraction of nucleotide-free

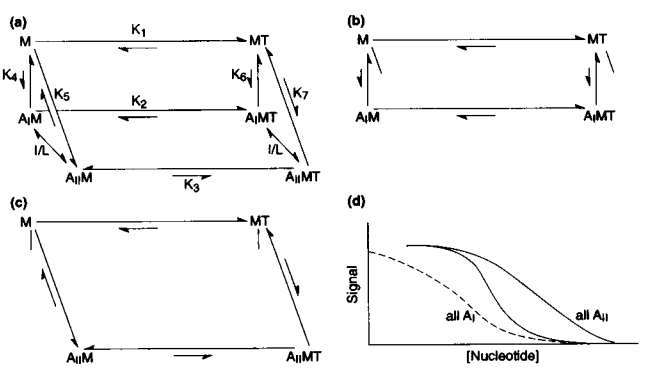

FIGURE 3 (*a*) A model explaining the different affinities of nucleotide binding to cross-bridges depending on the state of the thin filament. M, S1 of myosin; A_I, the inactivated form of the thin filament; A_{II}, the activated form of the thin filament; T, nucleotide. All constants K are association constants. $1/L$ is the equilibrium constant between A_I and A_{II} forms in the absence of S1 and Ca^{2+} (Hill et al., 1981). (*b*) In the absence of Ca^{2+} (all actin in the A_I form), both nucleotide-bound and nucleotide-free myosin heads can only bind with low affinity to actin. Because of detailed balance, nucleotide affinity for both attached and detached myosin heads is high (qualitatively shown by length of arrows). (*c*) In the presence of saturating Ca^{2+}, all regulated units of actin are in the active form A_{II}. Nucleotide-free myosin heads bind to A_{II} with high affinity, whereas myosin heads with a nucleotide representing a weak analog (e.g., GTP) will still bind with low affinity to A_{II}. It follows from detailed balance that the nucleotide affinity for strongly attached cross-bridges (i.e., $A_{II}M$) is different (much less) than the nucleotide affinity for detached heads (M) and hence much less than that for cross-bridges attached to A_I. (*d*) In the absence of Ca^{2+}, as the nucleotide concentration decreases, the actin filament begins to be activated. This will result in a steeper change in the titration curve (*middle solid curve*) than that would be expected for a system in which the state of the thin filament would not change (all A_I).

attached myosin heads rises above ~40% (Greene and Eisenberg, 1980a). This will result in a steeper change in the titration curve (Fig. 3 *d*, *middle curve*) when nucleotide is reduced than would be expected for a system in which the state of the thin filament would not change ("all A_I" in Fig. 3 *d*).

In the presence of saturating Ca^{2+} let us assume that all regulated units of actin are in the active form (A_{II}; see Fig. 3 *c*). Nucleotide-free myosin heads bind to the active form of actin with high affinity, whereas myosin heads with a nucleotide representing a weak analog (e.g., GTP) will still bind with low affinity to actin. In such a system it follows from detailed balance that the nucleotide affinity for strongly attached cross-bridges is different (much less) than the nucleotide affinity for detached heads and hence much less than that for cross-bridges attached to the inactive form of actin. (If Ca^{2+} alone does not bring all regulated units of actin to the active form, the situation will be intermediate. However, as nucleotide concentration is reduced, an increasing number of regulated units will be fully activated.) The scheme shows that *as long as the strength of binding of nucleotide-free myosin heads to actin is different at low and high Ca^{2+}, and nucleotide·S1 binds to actin (A_I and A_{II}) weakly, detailed balance shows that nucleotide affinity for attached cross-bridges will be affected by the state of the thin filament.*

As an illustration, we apply Scheme I (Fig. 3 *a*) to interpret the present results. K_1 is independent of the state of the thin filament. If the cross-bridges are in weakly binding states, K_6 and K_7 are Ca^{2+} insensitive and similarly weak. Modeling of earlier binding data indicated that K_4 is similar to K_6 (Hill et al., 1980). K_5, however, is greater than K_4. According to detailed balance, $K_1K_6 = K_2K_4$ (in EGTA) and $K_1K_7 = K_3K_5$ (in CaEGTA). Since $K_6 \approx K_7$ (Fig. 1), then $K_2K_4 \approx K_3K_5$. Since $K_5 > K_4$, then $K_2 > K_3$. Table 2 lists the equilibrium constants obtained in solution, some of which are direct results, and some are extrapolated to higher ionic strength from solution studies of ATP · S1 and pPDM · S1 at different ionic strengths (Chalovich et al., unpublished results).

The original scheme of Hill et al., as used in Fig. 3, does not include two-step binding of myosin to actin (Trybus and Taylor, 1980; McKillop and Geeves, 1993). Fig. 4 *a* illustrates that our observations are also expected when two-step binding to actin is considered.

If all regulated units are in the inactive form (A_I; Fig. 4 *b*), the situation is the same as for the scheme shown in Fig. 3; again, nucleotide affinity is the same for detached myosin heads (M) and myosin heads attached to the inactive form of actin. Similarly, as the nucleotide is reduced, an increase in nucleotide-free myosin heads attached to actin will at some point begin to shift the actin to its active form (Fig. 4 *c*), where high-affinity binding can occur. When the actin units are in the active form (A_{II}), the relevant species for the present consideration would be $A_{II}M_h$ (high-affinity binding) and $A_{II}M_lT$ (low-affinity binding), with the ratio $A_{II}M_lT/A_{II}M_h$ perhaps less extreme than MT/M or $A_IMT/$

TABLE 2 Association constants, *K*, involving S1, actin, and various nucleotides obtained from solution studies

Association constants	ATP	ATPγS	GTP
K_1	10^{10-11} M^{-1*}	10^7 $M^{-1\#}$	10^7 $M^{-1\S}$
$K_2^{\|}$	10^{10} M^{-1}	10^7 M^{-1}	10^7M^{-1}
$K_3^{\|}$	10^6 M^{-1}	10^3 M^{-1}	10^3 M^{-1}
K_4**	10^3 M^{-1}	10^3 M^{-1}	10^3 M^{-1}
K_5##	10^7 M^{-1}	10^7 M^{-1}	10^7 M^{-1}
K_6	10^3 $M^{-1\P}$	10^3 $M^{-1\#}$	10^3 M^{-1}
K_7	10^3 $M^{-1\P}$	10^3 $M^{-1\#}$	10^3 M^{-1}

*Wolcott and Boyer (1975); Goody et al. (1977).

#Resetar and Chalovich (1995) (extrapolated to 170 mM ionic strength wherever possible).

§Assumed to be similar to K_1 for ATPγS, because K_6 and K_7 have been shown to be similar by binding data obtained in this study and that of Resetar and Chalovich (1995). K_3 for the two nucleotides appears to be similar from the x-ray diffraction data obtained by this study and that of Kraft et al. (1992).

¶Chalovich and Eisenberg (1982) (extrapolated from low-ionic-strength binding data).

‖Calculated by detailed balance.

**Based on modeling of binding data (Hill et al., 1980).

##Greene and Eisenberg (1980).

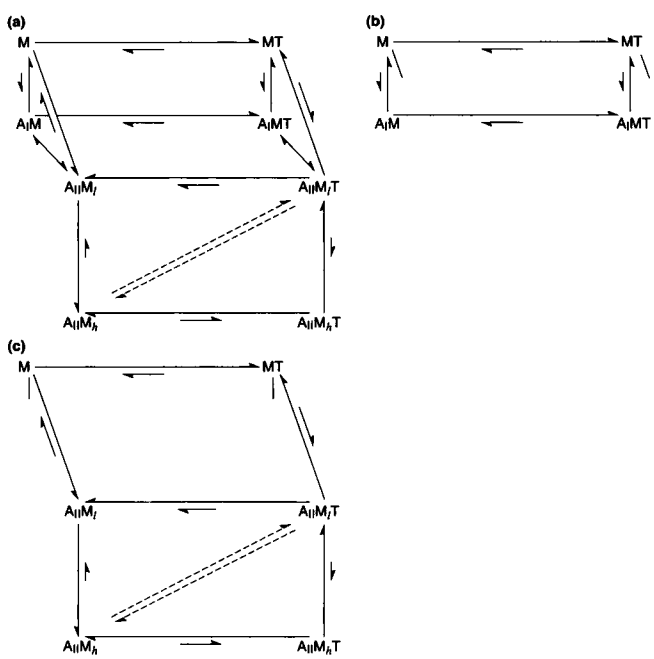

FIGURE 4 (*a*) A model similar to that in Fig. 3 *a*, except that two-step binding of myosin heads to actin is considered. M, S1 of myosin; A_I, the inactivated form of the thin filament; A_{II}, the activated form of the thin filament; T, nucleotide. M_l, low-affinity binding state of myosin; M_h, high-affinity binding state of myosin. (*b*) In the absence of Ca^{2+} (actin units in A_I form), the situation is the same as for the scheme shown in Fig. 3 *b*. Again, nucleotide affinity is the same for detached myosin heads (M) and myosin heads attached to A_I. (*c*) In the presence of Ca^{2+} (actin units in A_{II} form), the relevant species for the present consideration would be $A_{II}M_h$ and $A_{II}M_lT$, where the ratio $A_{II}M_lT/A_{II}M_h$ is perhaps less extreme than MT/M or A_IMT/A_IM, as shown in Fig. 3. Nevertheless, nucleotide affinity would still very much depend on the state of the actin filament (A_I versus A_{II}).

A_IM, as shown in Fig. 3. Nevertheless, nucleotide affinity would still very much depend on the state of the actin filament (A_I versus A_{II}).

The value of K_1 for GTP binding to S1 in Table 2 is estimated to be 10^7 M^{-1}. It is assumed to be similar to the K_1 for ATPγS, since K_6 and K_7 have been shown to be similar by the binding data obtained in this study and that of Resetar and Chalovich (1995); K_3 for the two nucleotides appears to be similar from the x-ray diffraction data obtained by this study and that of Kraft et al. (1992). It should be noted that an upward/downward estimate of K_1 will not change the relative magnitudes of K_2 and K_3, since the relative magnitudes of K_4 versus K_5 and K_6 versus K_7 are the determining factors.

The wide titration curves of ATPγS and GTP in the presence of high [Ca^{2+}] (Kraft et al., 1992; Fig. 2) are in fact expected for an ensemble of attached cross-bridges in a muscle fiber. Due to the mismatch between the periodicities of the myosin filaments and the actin filaments, the attached cross-bridges sustain a wide range of strains, resulting in a broad range of attachment/detachment equilibrium constants (Hill, 1974; Kuhn et al., 1994).

Considerations of ATP contamination and mixture of states

The interpretation presented above relies on S1·GTP being weak binding, which is supported by the present results. It could be argued, however, that there is a trace amount of ATP present that causes relaxation or activation (in the presence of Ca^{2+}). This appears highly unlikely. First, any contaminating ATP in our GTP solutions is below our level of detection. Second, during the course of the x-ray exposure, solutions containing EGTA or CaEGTA were alternately applied to the muscle fiber. If there was significant ATP contamination, activation would have occurred, and the striation pattern and x-ray diffraction pattern would have deteriorated. This was not observed. On the contrary, preliminary mechanical measurements showed no detectable force within experimental error (≤4% that of ATP) under activating conditions. No disordering of the striation pattern due to activation was observed under a microscope during the x-ray experiments, and the optical diffraction patterns of the sarcomere also did not show any evidence of activation.

Relation to previous work

A number of studies in the past have suggested that in the presence of Ca^{2+}, cross-bridges with ATPγS and GTP bind strongly to the thin filament (strongly bound, "pre-power stroke" state). This was based on the observation that upon addition of Ca^{2+} the fiber properties under study shifted toward rigor-like (e.g., an increase in stiffness (Dantzig et al., 1988; Pate et al., 1991, 1993; Wang et al., 1993) or a decrease in mobility of spin labels (Fajer et al., 1995)), but there was no active force being generated. The results of Kraft et al. (1992) and our present results suggest that in the presence of Ca^{2+}, incomplete saturation of cross-bridges with nucleotide can occur and give rise to the rigor-like states unless care is taken to ensure full saturation, e.g., at >10 mM ATPγS. In fact, preliminary results indicate that under saturating conditions of nucleotide (ATPγS), the layer line structures in the x-ray diffraction patterns are hardly distinguishable in the presence and in the absence of Ca^{2+} (Kraft et al., 1995b). Therefore, the ATPγS and GTP data do not provide evidence for the existence of some high-affinity and non-force-producing states at high [Ca^{2+}].

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