ENERGETICS OF THE ACTOMYOSIN BOND IN THE FILAMENT ARRAY OF MUSCLE FIBERS

EDWARD PATE* AND ROGER COOKE‡
*Department of Mathematics, Washington State University, Pullman, Washington 99164; and
‡Department of Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143

ABSTRACT The interaction between actin and myosin in the filament array of glycerinated muscle fibers has been monitored using paramagnetic probes and mechanical measurements. Both fiber stiffness and the spectra of probes bound to a reactive sulfydral on the myosin head were measured as the actomyosin bond was weakened by addition of magnesium pyrophosphate ($MgPP_i$) and glycerol. In the absence of $MgPP_i$, all myosin heads are attached to actin with oriented probes. When fibers were incubated in buffers containing $MgPP_i$, a fraction of the probes became disordered, and this effect was greater in the presence of glycerol. To determine whether the heads with disordered probes were detached from actin, spin-labeled myosin subfragment-1 (MSL-S1) was diffused into unlabeled fibers, and the fractions bound to actin and free in the medium were correlated with the oriented and disordered spectral components. These experiments showed that the label was oriented when MSL-S1 was attached to actin in a ternary complex with the ligand and that all heads with disordered probes were detached from actin. Thus the fraction of oriented labels could be used to determine the fraction of heads attached to actin in a fiber in the presence of ligand. The fraction of myosin heads attached to actin decreased with increasing [$MgPP_i$], and in the absence of glycerol ~50% of the myosin heads were dissociated at 3.3 mM ligand with little change in fiber stiffness. In the presence of 37% glycerol plus ligand, up to 80% of the heads could be detached with a 50% decrease in fiber stiffness. The data indicate that there are two populations of myosin heads in the fiber. All the data could be fit with a model in which one population of myosin heads (comprising ~50% of the total) sees an apparent actin concentration of 0.1 mM and can be released from actin with little change in fiber stiffness. A second population of myosin heads (~50%) sees a higher actin concentration (5 mM) and is only released in the presence of both glycerol and ligand.

INTRODUCTION

Muscle contraction results from a cyclic interaction between cross-bridges extending from the thick, myosin-containing filaments and binding sites on parallel actin filaments. Studies of myosin, actin, and nucleotide in solution have identified many kinetic intermediates of this interaction and much of their energetics. In the absence of nucleotide, actin and myosin form a tight complex. Binding of MgATP to the myosin nucleotide site results in a rapid dissociation of myosin from actin. Myosin with bound MgATP or hydrolysis products is thought to then form a weak bond with a new actin. MgADP and orthophosphate (P_i) are then released with an accompanying increase in the strength of the actomyosin bond, and the cycle begins again (for review, see Hibberd and Trentham, 1986; Cooke, 1986). A similar kinetic cycle is presumed to occur in the organized filament array of intact muscle fibers, and a fundamental question in the study of muscle biophysics is to understand the kinetics and energetics of this interaction in intact fibers and their relationships to force production.

Upon depletion of MgATP, active muscle enters a rigor state characterized by high tension and mechanical stiffness. Although nonphysiological in origin, there has been considerable interest in the rigor state as a model for those force-producing cross-bridges, which have reached the end of their working powerstrokes. The high affinity between actin and myosin in the absence of nucleotides has suggested that the formation of this bond plays some role in the chemomechanical energy transduction. Drawing upon solution studies, most models of cross-bridge kinetics postulate a weak bond at the beginning of the powerstroke, which becomes progressively stronger as the cross-bridge performs work, ending in the strong rigor bond at the end of the powerstroke (Eisenberg et al., 1980). This suggests that the strength of this bond should vary as the position between myosin and actin sites varies and that the energetics of the actomyosin bond determined in solution studies should be altered in the filament array of the fiber, where the mismatch between the periodic arrays of actin sites and myosin heads provides steric constraints on bond formation.

The actomyosin bond is greatly weakened by the binding of nucleotides or nucleotide analogues to the MgATP site on myosin. These states have been studied extensively both in solution and in fibers because they may represent states

that occur early in the powerstroke. In solution, the binding of MgATP or its two hydrolysis products weakens the bond by about a factor of 10^4, whereas the nonhydrolyzable nucleotide analogues, adenylyl-5'-imidodiphosphate (MgAMPPNP) or pyrophosphate ($MgPP_i$), produce states of intermediate strength (Greene and Eisenberg, 1980; Biosca et al., 1986; Konrad and Goody, 1982). Mechanically, binding of these nucleotide analogues is found to result in a change in both fiber tension and fiber stiffness (Marston et al., 1976; Schoenberg and Eisenberg, 1985; Brenner et al., 1986*a*). These changes could be due to either an actomyosin bond with altered mechanical properties or to cross-bridge detachment, or to both effects. The possibility of cross-bridge detachment has received support from combined stiffness and x-ray diffraction measurements in fibers (Brenner et al., 1986*b*) as well as proteolytic digestion experiments on myofibrils (Chen and Reisler, 1984; Assulin et al., 1986). Other studies using intact fibers, x-ray diffraction, and electron microscopy have clearly shown that MgAMPPNP produces changes in cross-bridge structure upon binding (Goody, et al., 1976; Lymm, 1975; Marston et al., 1976; Padron and Huxley, 1984; Reedy et al., 1983). These results have generally been interpreted to indicate new structural states in the presence of analogue. However, the possibility that some features of these patterns arise from myosin heads that have detached from actin has not been excluded.

One approach to the study of cross-bridge energetics is to use paramagnetic probes to measure cross-bridge orientation. These probes provide additional information and have several advantages over the above techniques (Barnett et al., 1986; Thomas, 1987). Previous work has shown that one can selectively and rigidly label a single sulfhydryl on the myosin heads with paramagnetic spin probes and then use electron paramagnetic resonance (EPR) spectroscopy to monitor changes in myosin head orientation and rotation. Using conventional EPR spectroscopy, Thomas and Cooke (1980) showed that in labeled fibers, the probes were highly oriented when myosin heads were bound to actin and were disordered when myosin heads were detached from actin. Binding of MgAMPPNP or $MgPP_i$ resulted in a shift from the ordered to the disordered fraction. This shift could result from myosin detachment, or it could arise from disordered myosin heads bound weakly to actin. The former interpretation is supported by saturation transfer EPR experiments, which measure the rotational correlation times of the probes. Saturation transfer EPR spectroscopy (ST-EPR) has shown that under a variety of conditions, there are two populations of heads: one with a rotational correlation time on the order of 10 μs, corresponding to detached heads, and the other with a correlation time greater than a millisecond, corresponding to attached heads (Thomas et al., 1980; Fajer et al., 1988; Barnett and Thomas, 1984). Thus spectroscopy of paramagnetic probes offers the potential for quantitative determination of the degree of cross-bridge dissociation in the intact filament array. Such measurements can provide a strong link between muscle physiology and the biochemistry of contractile proteins.

One method of measuring the strength of the actomyosin bond in the filament array of the fiber is to monitor the fraction of attached myosin heads under conditions that weaken the bond. As discussed above, the binding of a variety of ligands to the MgATP site on myosin weakens the actomyosin interaction. Several previous investigators have monitored the effect of these ligands on the actomyosin interactions in fibers and myofibrils. Ishiwata et al. (1985) measured cross-bridge motion, using ST-EPR spectra in myofibrils in the presence of $MgPP_i$ and MgAMPPNP. They reached the conclusion that these ligands dissociate myosin heads from actin. Brenner et al. (1986*a*) varied ionic strength in the presence of $MgPP_i$ and used fiber stiffness and x-ray diffraction patterns to estimate the fraction of attached cross-bridges. These workers defined an effective actin concentration in the millimolar range in the fibers.

Here we measure fiber stiffness and we estimate the fraction of myosin heads attached to actin using the spectra of paramagnetic probes attached to myosin. We first demonstrate that the spectra provide an accurate measure of the fraction of attached myosin heads, a measure that cannot be unambiguously obtained from x-ray diffraction patterns. We have studied an extensive range of conditions that greatly alter the strength of the actomyosin interaction. Ligand-induced dissociation of this bond is enhanced by high ionic strength, by low temperatures, and by a nonpolar solvent, ethylene glycol (Biosca et al., 1986; Greene and Eisenberg, 1980; Marston and Tregear, 1984; Konrad and Goody, 1982). We determined that $MgPP_i$ had a greater effect on probe spectra than did MgAMPPNP and that this effect was enhanced more by glycerol than by ethylene glycol. Thus we have investigated the responses of permeable, glycerinated, rabbit psoas fibers at high ionic strength (μ = 210 mM), 2°C, using varying concentrations of $MgPP_i$ and glycerol. To minimize the effects of the troponin–tropomyosin relaxing system upon myosin binding and EPR spectra, all experiments were done in the presence of saturating levels of calcium (pCa = 5).

The strength of the actomyosin bond in the fiber could be defined using the observed fraction of cross-bridges dissociated by $MgPP_i$ and glycerol, along with the affinity constants obtained in solution. The strength of this bond was found to vary, with at least two populations of myosin heads having different affinities for actin. Fiber stiffness was not a linear function of the fraction of disordered probes. Similar data are reported in the companion paper by Fajer et al. (1988) for muscle fibers bathed in MgAMPPNP. Both these studies suggest that fiber stiffness is not directly proportional to the fraction of attached myosin heads. However, stiffness may still be proportional to the number of attached cross-bridges, if one assumes

that each cross-bridge can be composed of a myosin with either one or two heads attached to actin.

METHODS

Thin strips of rabbit psoas muscle (1–2-mm diam) were dissected, tied to thin wooden rods with surgical thread, and glycerinated as described by Pate and Cooke (1985). To spin-label fibers, strips of glycerinated muscle were removed from sticks, dissected into small bundles (50–100 fibers), and labeled with *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperdinyl]maleimide (MSL) as described by Crowder and Cooke (1984). Myosin was prepared according to the method of Crooks and Cooke (1977) with myosin subfragment 1 subsequently prepared by chymotryptic digestion according to the protocol of Weeds and Taylor (1975). S1 was spin-labeled with MSL (MSL-S1) as described by Thomas and Cooke (1980). Hemoglobin was purchased from Sigma Chemical Co (St. Louis, MO) and labeled following the procedure of Boyens and McConnell (1966). Spin-labeled ATP, 2'-/3'-0-(1-oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine) adenosine 5'-triphosphate (SL-ATP) was supplied by Dr. Mark Crowder (Crowder and Cooke, 1987).

Experiments were done in a rigor buffer containing 0.18 M potassium acetate (KAc), 5 mM magnesium acetate, 1 mM EGTA, 0.95 mM $CaCl_2$, 20 mM 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES), pH 7.0, 2° or 20°C, pCa 5 with varying amounts of $MgPP_i$ and glycerol. As noted in Results, some experiments were done in the above buffer with a KAc concentration of 0.05 M at a temperature of 20°C. $MgPP_i$ concentrations were calculated using an affinity constant for Mg^{2+} of $2.5 \times 10^3\ M^{-1}$ (Smith and Martell, 1975).

EPR measurements were performed using an IBM Instruments, Inc. (Danbury, CT), ER 200 Series, EPR spectrometer. First derivative absorption spectra were obtained with the following instrument settings: microwave power, 25 mW; modulation, 0.8–2 Gauss at a frequency of 100 kHz. Each spectrum used in data analysis represents the average of between 5 and 25 distinct sweeps from an individual experimental preparation; sweep time was 10 s. To obtain muscle fiber EPR spectra, a TE011 cavity (Bruker Instruments, Inc., Billerica, MA) was used with a specially designed flat cell made of rexolyte. Small bundles of fibers (5-mm long) were incubated in the appropriate buffer, always allowing adequate time for diffusive equilibration (up to 6 h with some experiments involving S1). The fibers were then aligned in a well in the flat cell, carefully blotted to remove excess buffer, and covered with a rexolyte lid. Temperature was maintained by slowly blowing cooled nitrogen gas through the cavity and monitored by a small thermistor embedded in the rexolyte cell adjacent to the fibers. Fiber bundle volume was determined by weight. In experiments involving the comparison of spectra obtained from fibers with spectra from spin-labeled proteins in solution, the solution spectra were obtained by placing a known volume of buffer (5–10 μl) containing spin-labeled proteins into the flat cell.

The relative fractions of ordered and disordered probes attached to the SH-1 were determined as follows. A given peak height in P1 represents twice (2.0 ± 0.1) as many spins as does an equal peak height in P2 (peaks defined in Fig. 1). Thus the fraction of ordered probes is given as $P_2/(2P_1 + P_2)$. The value of this parameter for rigor fibers was taken to represent 100% ordered probes on the SH-1, and the value of this parameter for fibers incubated in rigor buffer with 0.5 M KAc and either 3.3 mM $MgPP_i$ or 4 mM MgATP at 2°C was taken as 0% ordered probes on the SH-1. The spectrum obtained in 0.5 M KAc was always the last spectrum in the series because the high ionic strength would dissolve some of the myosin filaments. The fraction of ordered probes attached to SH-1 in the presence of $MgPP_i$ was taken from a linear interpolation of this parameter between these two extremes. This protocol avoided problems introduced by some disordered probes that were not attached to SH-1, and by the fact that P2 is not quite zero even when all the heads are dissociated from actin.

For mechanical measurements, single fibers were dissected on a cold stage and mounted in a well between a solid state force transducer (Akers 801; Aksjelskapet Microelectronik, Horten, Norway) and an arm connected to a rapid motor (General Scanning, Inc., Watertown, MA) for changing muscle length. Duco cement (Dupont Co., Wilmington, DE) diluted 1:10 in acetone was used as a glue. Rigor buffer was added to the well in which the fiber rested, immersing the fiber. Temperature was maintained by passing cooled water through the aluminum block surrounding the well and monitored by a small thermistor adjacent to the force transducer. Mounted fiber length was 1 cm, and the resonant frequency of the transducer with mounted rigor fiber was 2 kHz. Sarcomere lengths varied between 2.4 and 2.6 μM as determined by laser diffraction. Additional details of the experimental apparatus are provided in Crowder and Cooke (1984). To determine fiber stiffness, a series of rapid extensions of muscle length of differing magnitude were applied to a fiber. A 1% change in muscle length was 90% complete in 0.5 ms. This corresponds to a speed of stretch of $\sim 2 \times 10^3$ nm/half sarcomere per s. A least-squares linear fit was made to the linear portion of a plot of peak force versus the percent length change. The slope was taken as fiber stiffness.

Some of the conclusions reached here depend on a comparison between fiber stiffness and EPR spectra. To minimize differences between the two measurements, each was done using the same solution and labeled fibers from the same preparation. In each case, the temperature was monitored by a thermistor close to the mounted fibers and varied by less than 0.5°C.

RESULTS

The basic phenomena to be observed using conventional EPR spectroscopy are shown in Fig. 1. Fig. 1 *A* is the spectrum obtained from a bundle of labeled muscle fibers oriented parallel to the magnetic field. EPR spectra from MSL-labeled muscle fibers have been extensively characterized previously (for review see Thomas, 1987). Spectra, such as those shown in Fig. 1, have been found to consist of two components. One population of probes is highly oriented, giving rise to three sharp lines. These probes are on myosin heads that are bound to actin. A second population is highly disordered with an approximately isotropic distribution, giving rise to broad peaks. This population has been identified with myosin heads that are detached from actin (discussed in more detail below). These two populations are most easily resolved by observ-

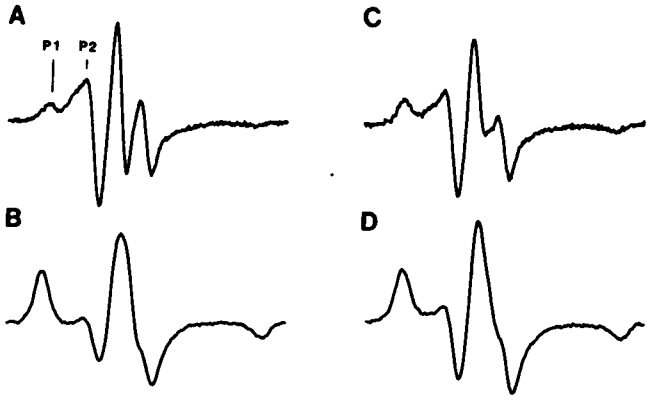


FIGURE 1 EPR spectra of glycerinated rabbit psoas fibers labeled at the reactive sulfhydryl with MSL. In this and subsequent spectra, the fibers are oriented parallel to the magnetic field, and the derivative of absorption is plotted as a function of the magnetic field. The width of each spectrum shown here is 0.9 mTesla. The fibers are bathed in rigor buffer, 2°C with (*A*) 0 $MgPP_i$, (*B*) 3.3 mM PP_i and 0.5 M KAc, (*C*) 3.3 mM $MgPP_i$ and (*D*) rigor buffer containing 37% glycerol and 3.3 mM $MgPP_i$.

ing the two low field peaks. In Fig. 1, P1 arises from the disordered distribution and P2 from the ordered distribution. The spectrum in Fig. 1 *B* results when the fibers in Fig. 1 *A* are subsequently incubated in rigor buffer containing 3.3 mM $MgPP_i$ and a high concentration of salt (0.5 M KAc). Under these conditions, all myosin heads are dissociated from actin, fiber stiffness is close to zero, and all probes are disordered. Spectrum 1 *B* is identical to that obtained from a fully relaxed fiber (Thomas and Cooke, 1980). Spectrum 1 *C* shows that the presence of 3.3 mM $MgPP_i$ in rigor buffer results in a marked shift of probes from the ordered to the disordered fraction as previously shown (Thomas and Cooke, 1980). If the nonpolar solvent glycerol is added to the medium, $MgPP_i$ produces a greater effect as shown in Fig. 1 *D*.

In each set of fibers investigated, spectra were obtained under the conditions of Fig. 1, *A* and *B* to define the spectra for all myosin heads attached to, or detached from actin, respectively. The spectra obtained under other conditions were found to be a linear combination of these two spectra, and the fraction of ordered probes attached to SH-1 was obtained by a linear extrapolation between Spectrum 1 *A* (100% ordered probes) and Spectrum 1 *B* (0% ordered probes), as described in Methods.

Do Ternary Complexes with Disordered Probes Exist?

Previous results have led to the conclusion that under rigor conditions, in the absence of nucleotide analogues, the ordered fraction represents myosin heads bound to actin in the rigor state whereas the disordered fraction results from the disordered orientations assumed by myosin heads that are dissociated from actin (Thomas and Cooke, 1980; Barnett and Thomas, 1984). Both $MgPP_i$ and nonpolar solvents weaken the acto–S1 bond in solution (Greene and Eisenberg, 1980; Marston and Tregear, 1984; Biosca et al., 1986). Thus the spectral shifts from an ordered to a disordered component induced by these agents are consistent with dissociation of myosin heads from actin in the fibers. The possibility exists, however, that the shift from the ordered fraction to the disordered fraction results not only from dissociation of the myosin head, but also from the presence of a weakly bound, ternary state in which the myosin head, or a portion of it, can assume random orientations. Subsequent interpretation of the data requires investigation of this possibility.

To verify that the change in EPR spectra does indicate detachment of heads from actin, we measured the spectra of MSL-S1 bound to fibers. Using the following protocol, we could independently measure the fractions of MSL-S1 that were attached to and detached from actin, and we compared these fractions to the spectral components. Unlabeled fibers were incubated in rigor buffer containing 50 μM MSL-S1 and 4 mM $MgPP_i$, and the magnitude of the disordered spectral component was compared to that expected for 50 μM MSL-S1 diffusing in the interfilament space. If a weakly attached, ternary complex contributes to the random component of the spectrum, these will not be equivalent. To make this comparison, we first found the volume available for solvating S1 in the aqueous component of the fiber bundle. To determine this volume, the intensity of the EPR signal obtained from a known volume of unlabeled muscle fibers, which had been equilibrated in rigor buffer containing 5 mg/ml MSL-labeled hemoglobin, was compared with the signal obtained from an identical volume of rigor buffer containing 5 mg/ml MSL-Hb. The ratio of the former to the latter, 0.64 ± 0.02 (mean ± SEM, 12 observations) determines the volume available for solvating macromolecules.

Fig. 2 shows a spectrum obtained from fibers incubated in the presence of 50 μM MSL-S1 in rigor buffer plus 4 mM $MgPP_i$ compared with the spectrum of free MSL-S1 with an intensity adjusted to represent unbound S1 diffusing freely in the available fiber volume. As is evident from comparison of the lowfield peaks (P_1), the random component in fibers incubated in MSL-S1 and $MgPP_i$ can be completely accounted for by detached MSL-S1 in the aqueous component of the fiber bundle matrix within experimental error. The ratio of the two components is 1.00 ± 0.04 (mean ± SEM, four observations).

The difference between the two spectra shown in Fig. 2 represents the spectrum of S1 heads bound to actin in the presence of $MgPP_i$. The conditions used in Fig. 2 result in a very low concentration of bound S1, making the difference spectrum noisy. To increase the amount of bound MSL-S1, enhancing the signal-to-noise characteristics of the spectrum a second protocol was used. Fibers were first incubated in rigor buffer containing 50 μM MSL-S1 until equilibrated; they were next incubated in rigor buffer

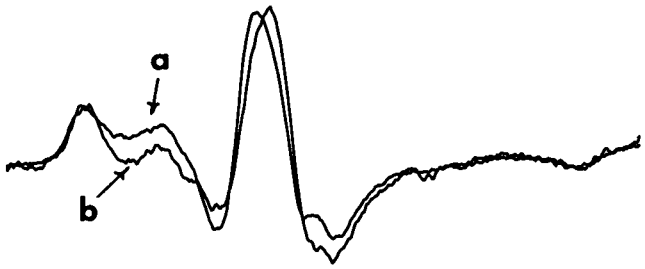


FIGURE 2 EPR spectrum of unlabeled fibers equilibrated with rigor buffer containing 50 μM MSL-S1, in the presence of 4 mM $MgPP_i$ (spectrum *a*) compared with that of free MSL-S1 in rigor buffer plus 4 mM $MgPP_i$ (spectrum *b*). The amplitude of the free MSL-S1 (spectrum *b*) has been adjusted to represent the expected spectrum of free MSL-S1 in the fiber. The adjustment was made by measuring the volume within the fiber that is available for solvating macromolecules using spin-labeled hemoglobin as described in the text. The equivalence of the amplitudes of the two low field peaks shows that the disordered component of the spectrum of the fiber equilibrated with MSL-S1 is completely accounted for by unbound MSL-S1. Temperature was 2°C.

containing 4 mM $MgPP_i$ allowing sufficient time for $MgPP_i$ to equilibrate, and they were then mounted in the flat cell before sufficient time elapsed for MSL-S1 to diffuse out. This protocol results in high concentrations of both bound and unbound S1 producing the spectrum shown in Fig. 3 *A*. To obtain the spectrum of bound MSL-S1, we assume that the random component of this spectrum is due to free S1. Fig. 3 *B* shows that the difference spectrum (MSL-S1 in fibers minus free MSL-S1) obtained is similar to that observed for S1 bound to rigor muscle in the absence of nucleotide analogues, shown in Fig. 1.

The spectrum of the ternary actin·S1·PP_i complex was also obtained under conditions in which the actomyosin bond is strong and little dissociation of myosin from actin occurs (rigor buffer with KAc reduced to 0.05M, 20°C). Fig. 4 shows the spectra with MSL-S1 obtained in the presence and absence of 4 mM $MgPP_i$. Addition of $MgPP_i$ causes little change, although, as shown below, more than 50% of the S1 has bound $MgPP_i$. If the average angle of the probe distribution had changed it would have altered the positions of the cross-over points shown by the arrows. A shift of 1 Gauss, which would be readily detectable, would arise from an angular change of 2°. This result thus leads to the conclusion that the ternary complex is ordered at the same orientation as rigor.

We conclude that in the presence of $MgPP_i$ there is no weakly attached actin·S1·PP_i state contributing to the disordered component of the spectrum, and thus the disordered component of the EPR spectrum is the result of detached S1 only. Furthermore, from the difference spectrum (Fig. 3 *B*) and the spectra in Fig. 4, we conclude that the EPR spectrum of the ternary complex is identical to that of the rigor state. No new angles are introduced.

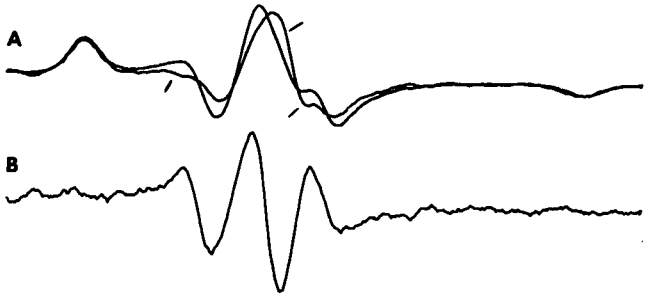


FIGURE 3 (*A*) EPR spectra of MSL-S1 bound to unlabeled fibers. To obtain a high internal concentration of bound MSL-S1, the fiber was first equilibrated with 50 μM MSL-S1 and subsequently incubated in rigor buffer containing 4 mM $MgPP_i$ for 2 min, sufficient time for $MgPP_i$ equilibration but for very little MSL-S1 to diffuse out. This spectrum is compared with that obtained from MSL-S1 in rigor buffer, 4 mM $MgPP_i$ labeled with tick marks. The amplitude of the spectrum of free MSL-S1 has been adjusted to equal the disordered spectral component of the spectrum of decorated fibers. (*B*) Difference spectrum between bound and unbound MSL-S1 from (*A*). This difference spectrum resembles the rigor spectrum (Fig. 1 *A*). showing that $MgPP_i$ has not altered the orientation of the probe.

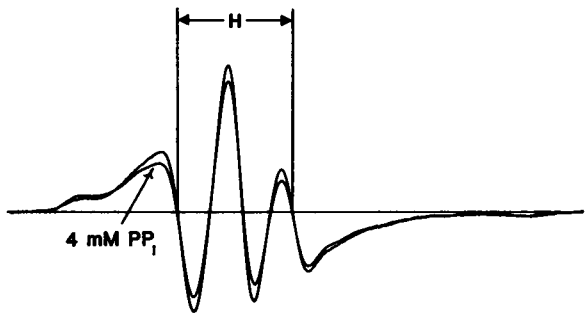
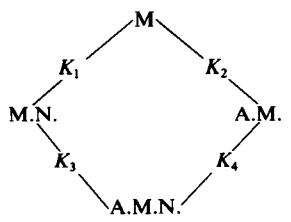


FIGURE 4 EPR spectra of unlabeled fibers decorated with MSL-S1 in rigor buffer with the KAc concentration reduced to 0.05 M, 20°C, with 0 and 4 mM $MgPP_i$. The spectrum in the presence of 4 mM $MgPP_i$ is identified by tick marks. Under these conditions the actomyosin bond is strong and $MgPP_i$ causes little dissociation of myosin from actin, and no observable change in the orientation of the ordered probes. Any change in the mean orientation of the ordered probes would have altered the splitting between the sharp lines, H, identified in the figure. A change in mean angle of 2° would alter H by 1 G, which would be easily measurable.

Measurements of Affinity Constants

Interpretation of the data in terms of the energetics of the actomyosin bond requires knowledge of the affinity constants for the actomyosin–ligand interaction in the presence and absence of 37% glycerol. Although some of these constants have been measured by previous investigators, they have not been evaluated in glycerol, and they are very dependent on temperature and ionic strength. Thus, we have reevaluated the affinity constants required for the analysis of the data under the conditions used here. The affinity constants are defined in the four-state model shown in Scheme I (Greene and Eisenberg, 1980) where A represents actin, M represents myosin or S1, and N represents the analogue, $MgPP_i$. Because both ligand and actin bind tightly to free myosin, the level of free myosin is negligible in our experiments, and the four-state model can be simplified to a three-state model that requires the specification of two affinity constants, K_3 and K_4.

The affinity of nucleotide for the actomyosin complex, K_4, was determined by competition between $MgPP_i$ and a paramagnetic derivative of MgADP (SL-ADP). The EPR spectrum obtained when SL-ADP is bound to the actomyosin complex in fibers is highly ordered (Crowder and Cooke, 1987). Fibers were incubated in rigor buffer containing 50 μM SL-ADP, and the spectrum obtained was



Scheme 1

compared with that obtained after addition of 4 mM $MgPP_i$ shown in Fig. 5. These spectra are the result of a complex superposition of immobilized spin-labels, resulting from SL-ADP bound to the actomyosin complex in fibers, in equilibrium with SL-ADP free in solution, as discussed in detail by Crowder and Cooke (1987). The spectrum from SL-ADP free in solution consists of three intense narrow peaks. The smaller peak to the left of the lowfield, narrow peak results from bound, immobilized SL-ADP. As shown in the figure, the peak is lower in the presence of 4 mM $MgPP_i$, reflecting the competition between ligands for the myosin nucleotide site. Addition of $MgPP_i$ resulted in a decrease in the amplitude of this peak of 50 ± 10%. This decrease in bound SL-ADP can be used to calculate the amount of bound $MgPP_i$. Using an affinity constant for SL-ADP to actomyosin of 7×10^3 M^{-1} (Crowder and Cooke, 1987) and a myosin concentration of 220 μM in fibers (Marston et al., 1976), K_4 is found to be 390 ± 30 M^{-1} (mean ± SEM, 6 observations). Repeating the same experiment using rigor buffer containing 37% glycerol, gives a value of 340 M^{-1}. Our basic conclusion is that K_4 is similar to that observed by Biosca et al. (1986), a value that is considerably weaker than previously suggested (Greene and Eisenberg, 1980; Konrad and Goody, 1982).

These values of K_4 were obtained at 20°C where little dissociation of the actomyosin complex occurs. The experimental determination of K_4 described in the preceeding paragraph is more complex at 2°C due to dissociation of a fraction of the myosin heads. However, the affinity constant of several nucleotides, including $MgPP_i$, for the actomyosin complex is not very temperature sensitive increasing by only a factor of 2 when the temperature is reduced from 20° to 2°C (Konrad and Goody, 1982; Greene and Eisenberg, 1980). The values given in Table I are adjusted to 2°C using this factor.

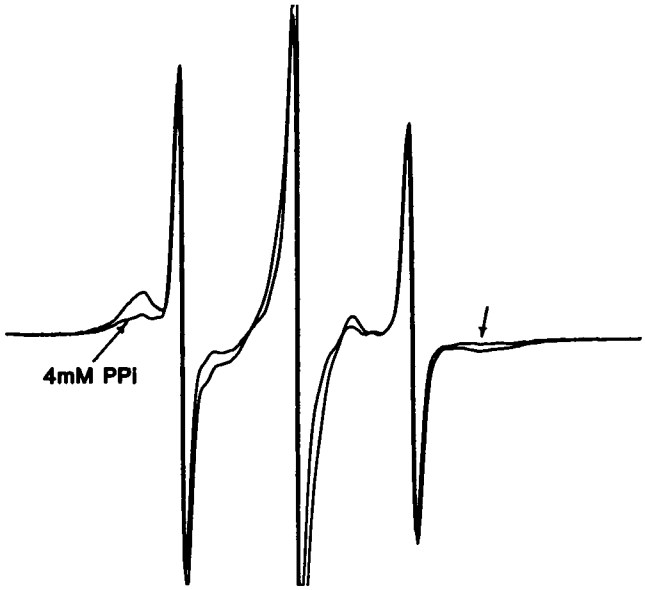


FIGURE 5 EPR spectra of unlabeled fibers in the presence of 50 μM SL-ADP rigor buffer, 20°C, with 0 and 4 mM $MgPP_i$. The three sharp peaks come from analogue that is not bound to protein. Broad peaks at low and high field are from SL-ADP bound to the nucleotide site. Addition of $MgPP_i$, which competes for the nucleotide site on myosin, decreases the intensity of the broad peaks due to bound SL-ADP by ~50%, allowing one to calculate the affinity of $MgPP_i$ for the actomyosin complex.

A value for K_3 was determined by measuring the fraction of MSL-S1 bound to unlabeled fibers and fitting the data with a model as described below. Incubation of fibers in MSL-S1 in the absence of $MgPP_i$ showed that the fibers contain 400 ± 50 μM (mean ± SEM, four observations) actin that is available for binding S1. Fibers were then incubated in buffer containing various concentrations of $MgPP_i$ with or without 37% glycerol. Under these conditions, some MSL-S1 could diffuse out from the fiber into solution. The results are shown in Fig. 6 where the upper curve is the data in the absence of glycerol and the lower curve is the data in 37% glycerol.

The EPR spectra provide a measure of the fraction of myosin heads attached to actin in the filament array of the fiber. To interpret these data in terms of the affinity of myosin for actin in the fiber, we have analyzed the data using the three-state approximation of the four-state model shown in Scheme 1. The fraction of bound heads is given by

$$\frac{\text{bound}}{\text{total}} = \frac{K_3A(1 + K_4N)}{K_3A + K_4N + K_3AK_4N}, \quad (1)$$

where A represents the actin concentration. Best fits of the data to Eq. 1 were determined by a nonlinear, least-squares fit of the $MgPP_i$ titration data using a Gauss-Newton type iteration (Dahlquist et al., 1974). All data points were weighted equally.

The fraction of S1 bound to actin in the fibers was measured using EPR spectra, and the concentration of free actin was determined from the difference betweeen the fraction of bound S1 and the maximum amount of S1 that bound in the absence of ligands. These data were fit to Eq. 1 to determine a value of K_3. The value of K_3 required to fit the data was greater at lower concentrations of ligand, varying linearly with the logarithm of MgPP: concentration from 6,400 M^{-1} at 100 μM $MgPP_i$ to 800 M^{-1} at 3.3 mM $MgPP_i$ in the absence of glycerol. A similar variation was seen in the presence of glycerol. The range of values found is given in Table I. The theoretical fits are shown by the solid lines in Fig. 6. The decrease in K_3 seen at the

TABLE I
AFFINITY CONSTANTS (M^{-1})

	K_3 Low ligand	K_3 High ligand	K_4
Rigor	6,400	800	800
Rigor + 37% glycerol	1,600	250	700

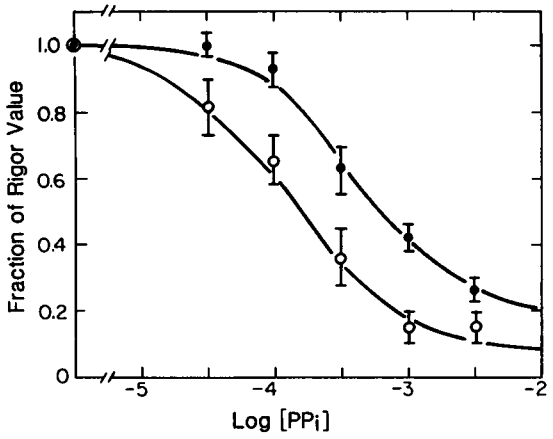


FIGURE 6 The fraction of ordered probes for MSL-S1 bound to unlabeled fibers as a function of $MgPP_i$ concentration. *Upper curve*, 0% glycerol; *lower curve*, 37% glycerol. Data are normalized with respect to the value in rigor buffer, in the absence of $MgPP_i$. Each data point is the mean ± SEM from a minimum of four observations. The solid lines are fits to the data using Eq. 1 as described in the text.

higher ligand concentrations is most probably due to the interaction between the myosin heads and the relaxing proteins. The variation seen here is similar to that observed for the binding of S1·AMPPNP to regulated actin in solution (Greene, 1982). Binding constants at 2°C, 0.18 M KAC rigor in the presence and absence of 37% glycerol are summarized in Table I.

Fraction of Myosin Heads Attached to Actin in Fibers

To investigate cross-bridge dissociation in the filament array, we have used MSL-labeled fibers and measured the fraction of disordered probes as a function of $MgPP_i$ concentration using a high ionic strength buffer, 2°C to maximize dissociation. The data, shown in Figs. 8 and 9, give the percentage of ordered probes, determined as described in Methods, normalized with respect to the rigor value. If the ordered probes measure the fraction of attached myosin heads, as found above, the data indicate increasing cross-bridge dissociation with increasing $MgPP_i$ concentration.

Rabbit psoas muscle contains an endogenous pyrophosphatase, which if present in sufficient quantity in the experimental preparation, would affect these results. Individual EPR spectra required up to 5 min to accumulate, allowing time for possible $MgPP_i$ hydrolysis. To investigate this possibility, fibers were incubated as above in MSL-S1 and 330 μM $MgPP_i$ with spectra taken at 0, 5, 10, and 15 min. The average change in the percentage of ordered probes was 1.2 ± 0.2%, showing that insufficient pyrophosphatase is present to affect our conclusions.

We have also measured muscle stiffness as a function of $MgPP_i$ concentration. Stiffness was measured by applying rapid length changes to fibers as described in Methods, plotting peak tension change following extension, and then using a least-squares fit over the linear portion of the plot to determine fiber stiffness as shown in Fig. 7. The data obtained over a range of $MgPP_i$ concentrations are shown in Fig. 8. No statistically significant difference was observed between labeled and unlabeled fibers. We observe that addition of up to 3.3 mM $MgPP_i$ causes little change in stiffness as has been reported previously for both $MgPP_i$ and MgAMPPNP (White, 1970; Marston et al., 1976; Kuhn, 1978). The 6% decrease we observe is less, however, than the 25% decrease observed by Brenner et al. (1986*a*, *b*) at a slightly higher, 4 mM, $MgPP_i$ concentration. This discrepancy appears to result from their use of chloride instead of acetate as the principal anion. With a rigor buffer containing 0.18 M KCl, we obtain a decrease in stiffness of 17 ± 4% (mean ± SEM, five observations) at 3.3 mM $MgPP_i$, roughly equivalent to that observed by Brenner and co-workers. In the presence of glycerol, $MgPP_i$ causes a disordering of the probes at a lower concentration, and also causes a substantial decrease in the stiffness. This is to be expected because the binding of actin to myosin is considerably weaker in the more nonpolar solvent. Using the data from Figs. 8 and 9, it is possible to construct a plot of fiber stiffness as a function of disordered probes, which is shown in Fig. 10. Assuming that ordered probes are a measure of attached mysoin heads, Fig. 10 shows that mechanical stiffness is not a linear function of the fraction of attached heads. Instead ~50% of the heads can be detached without significant decrease in fiber stiffness, with additional detachment resulting in decreased stiffness.

DISCUSSION

The strength of a protein–protein interaction is commonly measured in solution by varying the concentration of the reactants and measuring the fraction found in the complex. In the filament array of the muscle fiber, the concentrations of the reactants are fixed and this approach is not possible. We have circumvented this problem by varying the strength of the actomyosin interaction using ligands and solvents known to weaken the interaction, and by using

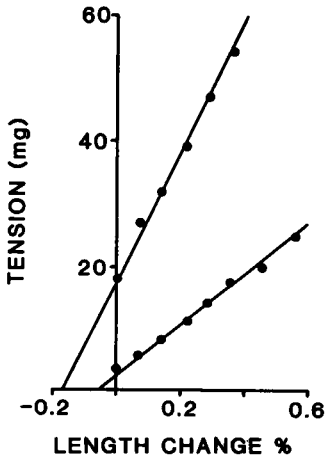


FIGURE 7 Peak force after a rapid length change as a function of fiber extension for MSL-labeled fibers in rigor buffer, 37% glycerol, 0 $MgPP_i$ (*upper curve*) and 3.3 mM $MgPP_i$ (*lower curve*). Least-squares fit over linear range gives fiber stiffness. The stiffness of the fibers in rigor buffer was 31 ± 5 N/mm^2, close to the value obtained by Tawada and Kimura (1984).

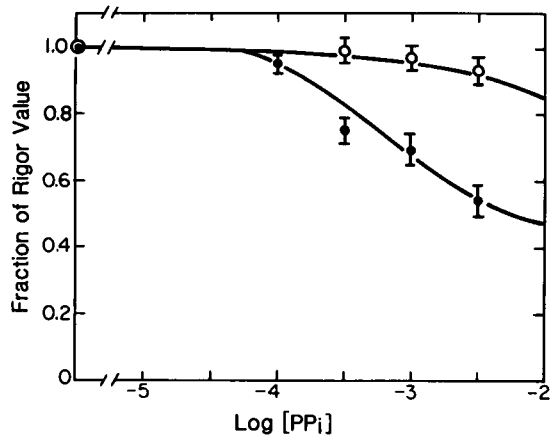


FIGURE 8 The fraction of ordered probes for MSL-labeled fibers (*lower curve*) and fiber stiffness (*upper curve*) are shown as a function of $MgPP_i$ concentration in rigor buffer, 2°C. Data are normalized with respect to rigor values. Mean ± SEM, minimum of four observations. The solid lines are fits to the data using Eq. 2 as described in the text.

paramagnetic probes to measure the fraction of myosin bound to actin. We first determined that paramagnetic probes bound to a reactive sulfydryl can be used to unambiguously measure the fraction of myosin heads attached to actin in the presence of $MgPP_i$. We then estimated the strength of the actomyosin bond by determining the concentrations of $MgPP_i$ and glycerol required to dissociate myosin from actin.

A common hypothesis for the events occurring during force generation holds that the myosin head initially binds weakly to actin in one orientation and subsequently changes to a second orientation in which the bond is very strong. The present work addresses several aspects of this hypothesis. We measured the affinity of myosin for actin to determine whether it has been affected by the filament array. In the accompanying paper, Fajer et al. (1988) investigate both conventional and ST-EPR spectra of

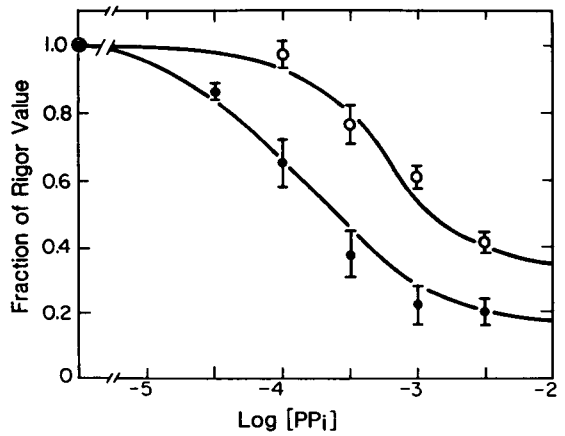


FIGURE 9 Percent ordered probes for MSL-labeled fibers (*lower curve*) and fiber stiffness (*upper curve*) as a function of $MgPP_i$ concentration in rigor buffer, 37% glycerol, 2°C, normalized with respect to rigor value. Mean ± SEM, minimum of four observations. The solid lines are fits to the data using Eq. 2 as described in the text.

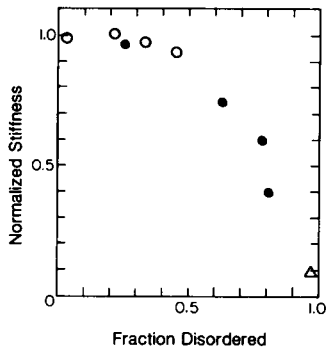


FIGURE 10 Stiffness (normalized with respect to rigor value) as a function of the fraction of disordered probes. Conditions: no glycerol (O); 37% glycerol, (●); 50% glycerol, (△). Data obtained in 0 and 37% glycerol taken from Figs. 8 and 9.

fibers incubated with MgAMPPNP. This analogue has been found by ourselves and others to resemble $MgPP_i$ in many respects, and the results found here for $MgPP_i$ are similar to those found for MgAMPPNP.

Are Probes Disordered in the Ternary Complex?

Interpretation of the spectra requires an identification of the spectral components with the state of the myosin head. Previous work has found that all spectra of MSL-labeled fibers can be decomposed into two components (Thomas and Cooke, 1980; Thomas et al., 1980; Barnett and Thomas, 1984; Ishiwata et al., 1985; Thomas, 1987). One component consists of three sharp lines, indicating that the probes are well-ordered with respect to the fiber axis. This spectral component is found in those fiber states in which myosin is known to be bound to actin, e.g., in rigor fibers, and is associated with a lack of rotational motion. The second component consists of broad lines indicative of probes that are highly disordered. This component is found in fibers in which myosin is unattached to actin, e.g., in relaxed fibers or fibers stretched beyond filament overlap, and is associated with a rotational correlation time of 1–10 μs. The identification of the ordered probes as indicative of an actomyosin bond appears straightforward; only the specific interaction with actin would be expected to produce this well defined orientation of the myosin head, and as discussed below, the orientation of these probes is not altered by the binding of $MgPP_i$. However, the identification of all disordered probes with heads that are not attached to actin in the presence of $MgPP_i$ is more ambiguous, and we first consider the possibility that some of the disordered probes are actually on myosin heads that are bound to actin. A resolution of these ambiguities, discussed in detail below, is crucial for interpreting the spectra.

A number of arguments support the conclusion that under the conditions employed here, the disordered probes are on myosin heads that are not attached to actin. The shape of the spectrum, which is indicative of a highly disordered, but not quite isotropic probe distribution is identical to that obtained in relaxed or stretched fibers when myosin heads are known to be detached from actin.

ST-EPR spectra show that both $MgPP_i$ and MgAMPPNP induce probe mobility in the microsecond time range (Ishitawa et al., 1985; Fajer et al., 1988). Thus the disordered probes appear to be undergoing large angle Brownian rotations identical to those found for relaxed fibers. In addition, the fraction of disordered or mobile probes is increased by conditions that favor dissociation of the actomyosin complex, e.g., lower temperature, higher ionic strength, or nonpolar solvents, as expected if these probes are on heads dissociated from actin.

The possibility that myosin heads may be attached to actin but still have probes that are disordered was investigated using MSL-S1. The aim of these experiments was to measure the spectrum of the MSL-S1 under conditions in which the fraction of attached and unattached heads could be measured independently. Fibers incubated in 50 μM MSL-S1 gave rise to spectra with a large disordered component and a smaller ordered component. At least a portion of the disordered component must arise from the MSL-S1 that is diffusing through the interfilament space, and to determine the magnitude of this portion we measured the volume of the fiber that is available for solvating macromolecules using spin-labeled hemoglobin. The available volume was found to be 64% in agreement with other studies. As shown in Fig. 2, the spectrum of free S1 scaled to represent the S1 that would be present in this volume exactly accounts for the spectral component arising from the disordered probes. In other words, there does not appear to be any S1 that is bound to actin with disordered probes. Fajer et al. (1988) reach identical conclusions from similar experiments with labeled S1 in the presence of AMPPNP. The fraction of such heads would have to be <20% of that with ordered probes to avoid detection. We make the assumption that this conclusion holds not only for S1, but also for myosin heads that are part of the filament array of the fiber. Ishiwata et al. (1985) measured the fraction of MSL-S1 bound to actin by sedimentation and found that it correlated well with the fraction of immobilized probes measured using ST-EPR. This result also supports the conclusion that disordered and mobile probes are on myosin heads that are detached from actin.

Several other observations also support the conclusion that $MgPP_i$ or MgAMPPNP dissociate myosin heads in muscle fibers. The reactivity of the SH-1 is high when myosin is detached from actin and is lowered upon attachment (Duke et al., 1976). Addition of $MgPP_i$ to fibers increases the specificity of probes for SH-1, as expected if the $MgPP_i$ has dissociated the myosin heads from actin. Attachment of myosin to actin also decreases the susceptibility to proteolysis of a region of the myosin head in the vicinity of residues 640–650. This susceptibility is partially restored in the presence of $MgPP_i$ or MgAMPPNP, with estimates of the fraction of heads dissociated in rough agreement with those found here and by Fajer et al. (Chen and Reisler, 1984; Assulin et al., 1986). $MgPP_i$ binds strongly to myosin ($K_d \simeq 0.5$ μM) but only weakly to the actomyosin complex, $K_d \simeq 2$–4 mM. These affinities are not substantially changed by the presence of nonpolar solvents. As shown in Fig. 9, when the actomyosin bond is weakened in the presence of glycerol, $MgPP_i$ produces a disordered component at a low concentration: 50% of the probes are disoriented at 100 μM $MgPP_i$. At 100 μM $MgPP_i$, very few myosin heads would bind ligand if the affinity of ligand for myosin were as weak as that of an actomyosin complex. As the opposite is the case, the heads with disordered probes must have a high affinity for the ligand, a property that is again similar to that of heads that are detached from actin. In a related experiment, Johnson (1986) measured the affinity of MgAMPPNP for myosin in myofibrils and came to very similar conclusions.

Summarizing the above discussion, data obtained from three locations on the myosin head—SH-1, residues 640–650, and the nucleotide binding site—all support the conclusion that the disordered component of the spectrum represents heads that are not attached to actin. One experimental observation has suggested that probes could be mobile in a ternary actin–myosin–ligand complex. Svensson and Thomas (1986) investigated myosin heads that were cross-linked to actin and found probe mobility in the presence of MgATP. Although this raises the possibility that probes could be mobile and disordered in an actin–myosin nucleotide complex, the question of whether the head remains rigidly bound to actin when cross-linked remains unresolved. More importantly for the conclusions drawn from our studies, Svensson and Thomas also correlated the fraction of immobile probes with the fraction of myosin heads that sedimented with actin. In the presence of MgAMPPNP or $MgPP_i$, these two fractions were the same, suggesting that these ligands did not induce probe mobility in the ternary complex (Svensson and Thomas, unpublished results). Hence we conclude that with the conditions used here, the spectral components can be used as a reasonable and quantitative measure of the fraction of myosin heads that are attached to actin. It remains a distinct possibility that this is not the case in the presence of MgATP.

The Orientation of the Ternary Complex

The orientation of the probes is not changed by the binding of $MgPP_i$. The lack of a change is most clearly evident in Fig. 4, where addition of 4 mM $MgPP_i$ has caused little change in the spectrum. As discussed below, over 50% of the myosin heads have bound $MgPP_i$ under these conditions. The binding of $MgPP_i$ to myosin weakens its affinity for actin by about three orders of magnitude. Such a change could reasonably be expected to alter the orientation of the myosin relative to the actin; however, the above result shows that no such change occurs in the vicinity of the reactive sulfhydryl. This conclusion is in line with previous studies of paramagnetic probes, which have also found that this site is not involved in the conformational

changes that must accompany force production (Cooke et al., 1982). Thus, if the binding of these ligands does cause any change in the conformation of the myosin head, this change must occur between the probe site and the junction with S2. An analysis of x-ray diffraction patterns also came to the conclusion that the binding of MgAMPPNP induced changes in the myosin head in a region that is close to the thick filament but not in that adjacent to actin (Padron and Huxley, 1984). This conclusion is compatible with the EPR spectra.

Strength of the Actomysin Bond in the Filament Array

The strength of the actomyosin interaction in the filament array can be estimated by comparing the ligand concentration required to dissociate heads in the fibers with that required to dissociate MSL-S1. The binding of ligand to the actomyosin complex was measured by competition with a spin-labeled analogue of MgADP. We found a value for K_4 of 700–800 M^{-1}, with little change induced by the presence of glycerol. A value for K_3 was obtained by fitting the data of Fig. 6 with Eq. 1. The release of the heads from the actin is governed by a competition between the magnitudes of K_4N and K_3A. Given the value of K_4 determined above and the concentration of total available actin determined from the binding of S1, a value for K_3 can be determined from the fit, leading to a value of 800 M^{-1} in the absence of glycerol and 250 M^{-1} in the presence of 37% glycerol. There has been considerable controversy over the magnitude of both K_3 and K_4, and our values are consistent with the weak ones recently obtained by Biosca et al. (1986). Our value for K_3 is also close to the apparent binding constant obtained from the inhibition of fiber contraction velocity (Pate and Cooke, 1985). Assuming a value for K_1 of 2×10^6 M^{-1} (Greene and Eisenberg, 1980), the value of K_2 can be evaluated by detailed balance ($K_1 \cdot K_3 = K_2K_4$). The value of K_2 determined in the absence of glycerol agrees well with that obtained by others when adjusted for the conditions and the presence of the relaxing proteins (Highsmith, 1977; Eisenberg and Greene, 1980; Konrad and Goody, 1980; Williams and Greene, 1983). The difference between K_3 in the presence and absence of glycerol, a factor of 4, is similar to that obtained by Marston and Tregear (1984) for ethylene glycol and MgAMPPNP.

While the binding of S1 to actin in solution is a second-order reaction, the binding of a myosin head to actin in the fiber is a first-order isomerization. Accepting the above arguments, the EPR spectra of labeled fibers allow us to measure the ratio of detached cross-bridges (M plus M·N states) to attached cross-bridges (A·M plus A·M·N states). We have analyzed the data of Figs. 8 and 9 assuming that the eight affinity constants given for Scheme I in Table I also hold in the fiber, but that the effective actin concentration (A_{eff}) "seen" by a myosin head is altered by the steric constraints of the filament lattice.

The data of Figs. 8 and 9 show that $MgPP_i$ is more effective in disordering the probes than in decreasing fiber stiffness. In both the absence and presence of glycerol, the fraction of disordered probes increases more rapidly than stiffness decreases. Efforts were first made to fit these data with Eq. 1, representing a single population of heads. Two simple assumptions relating stiffness to the fraction of attached heads can be made. If each head contributes equally to fiber stiffness, then stiffness should coincide with the fraction of ordered (attached) heads. This is clearly not the case. Alternatively one can assume that dissociation of both heads of a myosin molecule is required to affect fiber stiffness. Using this assumption and our values of K_3 and K_4, one effective actin concentration could not be obtained to fit simultaneously both the fiber stiffness and the fraction of ordered heads. This model predicts that dissociation of 50% of the myosin heads should result in a decrease in muscle stiffness to 75% of the rigor value. In contrast to this prediction, little change is seen in stiffness at the point where 50% of the heads are disordered.

The data shown in Figs. 8 and 9 suggests that there are two populations of heads in the fiber: one that binds more weakly to actin and does not contribute to fiber stiffness and one that binds more strongly and does contribute to stiffness. Because 50% of the myosin heads can be dissociated without a large change in fiber stiffness, we initially assumed that each of the two populations contains 50% of the myosin heads. The data as a function of $MgPP_i$ concentration were fit as follows to determine effective actin concentrations:

$$\frac{\text{bound}}{\text{total}} = F_1 \frac{K_3A_1(1 + K_4N)}{K_3A_1 + K_4N + K_3A_1K_4N} + F_2 \frac{K_3A_2(1 + K_4N)}{K_3A_2 + K_4N + K_3A_2K_2N} \quad (2)$$

Using relative fractions, F_1 and F_2, equal to 0.5, the ratio of attached myosin heads to detached myosin heads in both 0% and 37% glycerol were fit using Eq. 2 with appropriate K_3 and K_4 from Table I. The value of K_3 was assumed to depend on the fraction of attached myosin heads in the same fashion as it did on the fraction of attached MSL-S1. Since only the more strongly bound fraction is assumed to contribute to fiber stiffness, the stiffness data in 0% and 37% glycerol were correspondingly fit using Eq. 2 with F_1 equal to zero, F_2 equal to 1. The least-squares fit to Eq. 2 in which the effective actin concentrations, A_1 and A_2, were the variable, fitting parameters, indicates that all of the data shown in Figs. 8 and 9 can be fit with a model involving two equal populations of heads, one that sees an A_{eff} of 0.1 mM and does not contribute to fiber stiffness (A_1) and one that sees an A_{eff} of 5 mM and is stiff (A_2). The fit to the data was very sensitive to the value of K_3, and

slightly better fits were obtained (chi-square ~50% less) if the affinity constant K_3 in glycerol from Table I was adjusted down by a factor of 2.5. Importantly, the two values of A_{eff} were not changed by this modification. The fits define two values of A_{eff}, i.e., the effective actin concentrations seen by the two populations of heads in the fiber. The value of A_{eff} obtained here for the heads that are stiff (5 mM) is similar to the value obtained from the dependence of fiber stiffness on ionic strength (Brenner et al., 1986*b*). Similar conclusions were also drawn by Fajer et al. (1988) from studies employing MgAMPPNP.

Several attempts were made to fit the data using other models. Least-squares fits as above except that four parameters, F_1, F_2, A_1, and A_2, were allowed to vary in the fitting procedure indicate that the data do not allow the population of stiff heads to greatly exceed 50%. It could be smaller than 50%, however, and reasonable fits were obtained with this population comprising as few as 25% of the total heads. The data do not provide any evidence of a subpopulation of myosin heads that see an effective actin concentration >5 mM. When the glycerol concentration is raised from 37 to 50%, decreasing K_3 by about another factor of 7, only 10% of fiber stiffness remains, which is almost exactly that predicted by the three-state model described above. Thus any population of more tightly bound heads would have to be <10% of the total.

That the data can be fit with two equal populations of heads suggests that each population represents one head of the two-headed myosin molecule. The binding of one head of a myosin molecule weakens the binding of the second head, probably because of steric constraints imposed by the common connection at their distal end. The affinity of the second head for actin can be estimated from the relative affinities of S1 and heavy meromyosin (HMM) for actin in solution. The affinity of HMM is approximately equal to 2 times the affinity of S1 squared times the effective actin concentration seen by the second head. Using the data of Greene (1981), the effective actin concentration seen by the second head can be estimated to be 0.1 mM. Thus the population of weakly bound heads in the fiber has an affinity for actin that is similar to that of the second head of HMM estimated by comparison of the binding of HMM and S1 to actin in solution. The relationship between stiffness and the fraction of attached myosin heads can be explained if the attachment of one head to actin generates the full stiffness of the cross bridge. This would be the case if the compliant element is located in the segment of myosin that connects the myosin head to the thick filament. This model is strengthened by other structural data discussed below. Furthermore, the binding of spin-labeled HMM to actin by one head in the presence of MgADP has been observed in solution (Manuck et al., 1986).

The data discussed above as well as that of Fajer et al. (1988) reach the conclusion that at low temperatures and high concentrations of either $MgPP_i$ or MgAMPPNP, ~50% of the myosin heads are dissociated from actin. This conclusion would affect the interpretation of structural results from both electron microscopy and x-ray diffraction obtained under similar conditions. Diffraction patterns have shown that MgAMPPNP decreases the intensity of the actin-based layer lines without causing a large increase in many of the myosin-based layer lines. Such an effect could be explained if in the presence of $MgPP_i$ one head of each myosin were dissociated from actin with the remaining bound head preventing the dissociated head from association with the thick filament. Dissociation of both heads would be required for the association with the thick filament to occur. Such a possibility was considered by Padron and Huxley (1984) and by Lymm (1975). This model could also explain the results of Brenner et al. (1986*b*), who measured the changes in the ratio of the first two equatorials as a function of $MgPP_i$ and found that they paralleled the change in fiber stiffness. This observation would be expected if a single head detaches and remains in the vicinity of the thin filament. Electron micrographs of insect flight muscle taken in the presence of MgAMPPNP at low temperatures show that most cross-bridges are still formed in the presence of MgAMPPNP but that they have a different appearance from those seen in rigor (Reedy et al., 1983). That most cross-bridges are still formed shows that at least one head of each cross-bridge is still attached, lending support to the hypothesis that the population of heads shown to be dissociated by EPR represents the weakly bound head of each molecule. The detachment of one head would alter the appearance of the cross-bridge while leaving unchanged the total number of cross-bridges observed.

A currently popular hypothesis in the study of muscle physiology is that mechanical stiffness is proportional to, and thus a measure of, the number of attached cross-bridges. This conclusion is supported by the observation that fiber stiffness changes linearly with the amount of thick and thin filament overlap (Ford et al., 1981), and it correlates with fiber tension during fiber activation. Accepting the equivalence of disordered probes and detached myosin heads, Fig. 10 leads to the conclusion that mechanical stiffness is not proportional to the number of attached myosin heads under the conditions of these experiments. The compliance measured in these experiments is a combination of cross-bridge compliance and compliance due to elastic elements in series with the cross-bridges such as at the ends of the fiber where the fiber is attached to the tensiometer. This problem, however, does not alter the conclusion drawn above. If measured compliance does not change, then cross-bridge compliance also must not have changed. An investigation of the compliance of rigor, glycerinated fibers as a function of sarcomere length reached the conclusion that cross-bridges were the major source of compliance (Tawada and Kimura, 1984). If the conclusion that the more weakly bound head of each myosin molecule dissociates first is correct, and if a cross-bridge is defined as being made

when either one or both of the myosin heads are bound to actin, then stiffness could still be proportional to the fraction of cross-bridges bound. The data of Fig. 10 also help reconcile two apparently contradictory observations. Measurements of the stiffness of active muscle found a value that is 75% of that in rigor whereas the spectra of paramagnetic probes suggests that only 25% of the heads are attached to actin in active muscle (Goldman and Simmons, 1977; Cooke et al., 1982). As shown in Fig. 10, 75% of fiber stiffness is provided when only 35% of the heads are bound to actin.

Several conclusions concerning the energetics of the actomyosin bond can be drawn from the data presented. Different populations of heads appear to have different affinities to actin. The range of affinities is not great, however, and all myosin heads can make reasonably strong bonds with actin despite the steric constraints of the filament array. In fact, the energetics of the binding of myosin heads to actin in the filament array of the fibers is not much different from the binding of HMM to actin in solution. The effective actin concentration found in these experiments for the heads that bind more strongly to actin, 5 mM, is less than a factor of 10 different from the actual actin concentration of 0.6 mM. Thus the lattice constraints have strengthened the actomyosin bond by at most 6 kJ/mol. In many models of cross-bridge energetics, the bond between actin and myosin is weak at the beginning of the powerstroke and strong at the end of the powerstroke. In these models, energy transduction involves an interchange between a strong bond between myosin and actin and a strong bond between myosin and ATP. For these models to operate efficiently, the strength of the rigor, actomyosin bond at the end of the powerstroke must be an appreciable fraction (>50%) of the energy derived from ATP hydrolysis. Because the data obtained above allow us to calculate the strength of the actomyosin bond in the filament array, we can determine whether these models could operate with sufficient efficiency to explain that observed in living fibers. This comparison must be done at physiological conditions where muscle is expected to operate with high efficiency. Extrapolating the value of K_2 measured here to 37°C using the data of Konrad and Goody (1982) and Highsmith (1976), one obtains 4×10^7 M^{-1}. At an actin concentration of 5 mM, the formation of the bond between actin and one head of myosin would liberate ~30 kJ/mol of free energy, which is only a little less than that required for efficient energy transduction, which can be estimated from the efficiency of muscle contraction to be ~35–40 kJ/mol. Our results thus suggest that these models, which involve alternate strong bonds between actin·myosin and ATP·myosin, could operate efficiently.

The authors thank Drs. Dave Thomas, Peter Fajer, Mark Schoenberg, and Anthony Baker for helpful discussions of this manuscript. We also thank Kathy Franks-Skiba for a generous supply of S1 and Richard Fallon for typing the manuscript.

This work was supported by grants from the U. S. Public Health Service (AM30868) and National Science Foundation (DCB8511082).

Received for publication 23 June 1987 and in final form 13 November 1987.

REFERENCES

Assulin, O., J. Borejdo, and C. Flynn. 1986. Actin-attached and detached crossbridges in myofibrils: segregation into two populations according to their sensitivity to proteolytic digestion of myosin heavy chains. *J. Muscle Res. Cell Motil.* 7:167–178.

Barnett, V. A., and D. D. Thomas. 1984. Saturation transfer EPR of spin-labeled muscle fibers: dependence on sarcomere length. *J. Mol. Biol.* 179:83–102.

Barnett, V. A., P. Fajer, C. F. Polnaszek, and D. D. Thomas. 1986. High resolution detection of muscle cross-bridge orientation by electron paramagnetic resonance. *Biophys. J.* 49:144–146.

Biosca, J. A., L. E. Greene, and E. Eisenberg. 1986. Binding of ADP and ATP analogues to cross-linked and non-crosslinked acto.S1. 1986. *J. Biol. Chem.* 261:9793–9800.

Boyens, J. C. A., and H. M. McConnell. 1966. Spin-labeled hemoglobin. *Proc. Natl. Acad. Sci. USA.* 56:22–25.

Brenner, B., J. M. Chalovich, L. E. Greene, E. Eisenberg, and M. Schoenberg. 1986*a*. Stiffness of skinned rabbit psoas fibers in MgATP and $MgPP_i$ solution. *Biophys. J.* 50:685–691.

Brenner, B., L. C. Yu, L. E. Greene, E. Eisenberg, and M. Schoenberg. 1986*b*. Ca^{2+}-sensitive cross-bridge dissociation in the presence of magnesium pyrophosphate in skinned rabbit psoas fibers. *Biophys. J.* 50:1101–1108.

Chen, T., and E. Reisler. 1984. Tryptic digestion of rabbit skeletal myofibrils: an enzymatic probe of myosin cross-bridges. *Biochemistry.* 23:2400–2407.

Cooke, R. 1986. The mechanism of muscle contraction. *CRC Crit. Rev. Biochem.* 21:53–118.

Cooke, R., M. S. Crowder, and D. D. Thomas, 1982. Orientation of spin labels attached to cross-bridges in contracting muscle fibers. *Nature (Lond.).* 300:776–778.

Crooks, R., and R. Cooke. 1977. Tension generated by threads of contractile proteins. *J. Gen. Physiol.* 69:37–55.

Crowder, M. S., and R. Cooke. 1984. The effect of myosin sulphydryl modification on the mechanics of fiber contraction. *J. Muscle Res. Cell Motil.* 5:131–146.

Crowder, M. S., and R. Cooke. 1987. The orientation of spin-labelled nucleotides bound to myosin in glycerinated muscle fibers. *Biophys. J.* 51:323–333.

Dahlquist, G., A. Bjorck, and N. Anderson. 1974. Numerical Methods. Prentice-Hall, Inc., Englewood Cliffs, NJ. 821–824.

Duke, J., R. Takashi, K. Ue, and M. F. Morales. 1976. Reciprocal reactivities of specific thiols when actin binds to myosin. *Proc. Natl. Acad. Sci. USA.* 73:302–320.

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

Fajer, P. G., E. A. Fajer, N. J. Brunsvold, and D. D. Thomas. 1988. Effects of AMPPNP on the orientation and rotational dynamics of spin-labeled myosin heads in muscle fibers. *Biophys. J.* 53:513–524.

Ford, L. E., A. F. Huxley, and R. M. Simmons. 1981. The relation between stiffness and filament overlap in stimulated frog muscle fibers. *J. Physiol. (Lond.).* 311:219–249.

Goldman, Y. E., and R. M. Simmons. 1977. Active and rigor muscle stiffness. *J. Physiol. (Lond.).* 269:55P.

Goody, R. S., K. C. Holmes, H. G. Mannherz, J. Barrington Leigh, and G. Rosebaum. 1976. Cross-bridge conformation as revealed by x-ray diffraction studies of insect flight muscle with ATP analogues. *Biophys. J.* 15:687–705.

Greene, L. E. 1981. Comparison of the binding of heavy meromyosin and myosin subfragment 1 to F-actin. *Biochemistry.* 20:2120–2126.

Greene, L. E. 1982. The effect of nucleotide on the binding of myosin subfragment 1 to regulated actin. *J. Biol. Chem.* 257:13993–13999.

Greene, L. E., and E. Eisenberg. 1980. Dissociation of the actin subfragment 1 complex by adenyl-5'-yl imidodiphosphate, ADP, and PP_i. *J. Biol. Chem.* 255:543–548.

Hibberd, M. G., and D. R. Trentham. 1986. Relationships between chemical and mechanical events during muscular contraction. *Annu. Rev. Biophys. Biophys. Chem.* 15:119–161.

Highsmith, S. 1976. Interaction of the actin and nucleotide binding sites on myosin subfragment 1. *J. Biol. Chem.* 251:6170–6172.

Highsmith, S. 1977. The effect of temperature and salts on myosin subfragment-1 and F-actin association. *Arch. Biochem. Biophys.* 180:404–408.

Ishiwata, S., B. A. Manuck, J. C. Seidel, and J. Gergley. 1985. Saturation transfer electron paramagnetic resonance studies of attachment of myosin heads in myofibrils. *Biophys. J.* 49:821–829.

Johnson, R. E. 1986. Effect of ethylene glycol and Ca^{2+} on the binding of Mg^{2+}-adenyl-5'-yl imidodiphosphate on rabbit skeletal myofibrils. *J. Biol. Chem.* 261:728–732.

Konrad, M., and R. S. Goody. 1982. Kinetic and thermodynamic properties of the ternary complex between F-actin, myosin subfragment 1 and adenosine 5'-[β, γ-imido] triphosphate. *Eur. J. Biochem.* 128:547–555.

Kuhn, H. J. 1978. Cross-bridge slippage induced by the ATP analogue AMP-PNP and stretch in glycerol-extracted fibrillar muscle fibers. *Biophys. Struct. Mech.* 4:159–168.

Lymm, R. W. 1975. Low angle x-ray diagrams from skeletal muscle: the effect of AMPPNP, a nonhydrolyzed analog of ATP. *J. Mol. Biol.* 99:567–582.

Manuck, B. A., J. C. Seidel, and J. Gergely. 1986. Single-headed binding of a spin-labeled-HMM-ADP complex to F-actin. *Biophys. J.* 50:221–230.

Marston, S. B., and R. T. Tregear. 1984. Modification of the interactions of myosin with actin and 5'-adenylyl imidodiphosphate by substitution of ethylene glycol for water. *Biochem. J.* 217:169–177.

Marston, S. B., C. D. Roger, and R. T. Tregear. 1976. Changes in muscle crossbridges when β, γ-imido ATP binds to myosin. *J. Mol. Biol.* 104:263–276.

Padron, R., and H. E. Huxley. 1984. The effect of the ATP analog MgAMPPNP on the structure of crossbriges in vertebrate skeletal muscles: x-ray diffraction and mechanical studies. *J. Muscle Res. Cell Motil.* 5:613–655.

Pate, E., and R. Cooke. 1985. The inhibition of muscle contraction by adenosine 5' (β,γ-imido)triphosphate and by pyrophosphate. *Biophys. J.* 47:773–780.

Reedy, M. C., M. K. Reedy, and R. S. Goody. 1983. Co-ordinated election microscopy and x-ray studies of glycerinated insect flight muscle. II. Electron microscopy and image reconstruction of muscle fibers in rigor, in ATP and in MgAMPPNP. *J. Muscle Res. Cell Motil.* 4:55–81.

Schoenberg, M., and E. Eisenberg. 1985. Muscle cross-bridge kinetics in rigor and in the presence of ATP analogues. *Biophys. J.* 48:863–871.

Smith, R. M., and A. E. Martell. 1975. Critical Stability Constants. Plenum Publishing Corp., New York. Vol. 4 p 59.

Svensson, E., and D. D. Thomas. 1986. ATP induces microsecond rotational motions of myosin heads crosslinked to actin. *Biophys. J.* 50:999–1002.

Tawada, K., and M. Kimura. 1984. The stiffness of glycerinated rabbit psoas fibers in the rigor state. *Biophys. J.* 45:593–602.

Thomas, D. D. 1987. Spectroscopic probes of muscle cross-bridge rotation. *Annu. Rev. Physiol.* 49:691–709.

Thomas, D. D., and R. Cooke. 1980. Orientation of spin-labeled myosin heads in glycerinated muscle fibers. *Biophys. J.* 32:891–906.

Thomas, D. D., J. C. Seidel, J. S. Hyde, and J. Gergley. 1975. Motion of subfragment-1 in myosin and its supramolecular complexes: saturation transfer electron paramagnetic resonance. *Proc. Natl. Acad. Sci. USA.* 72:1729–1733.

Thomas, D. D., S. Ishiwata, J. C. Seidel, and J. Gergley. 1980. Submillisecond rotational dynamics of spin-labeled crossbridges in myofibrils. *Biophys. J.* 32:873–890.

Weeds, A. G., and R. S. Taylor. 1975. Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin. *Nature (Lond.).* 257:54–56.

White, D. C. S. 1970. Rigor contraction and the effect of various phosphate compounds on glycerinated insect flight and vertebrate muscle. *J. Physiol. (Lond.).* 208:583–605.

Williams, D. L., and L. E. Greene. 1983. Comparison of the effects of tropomyosin and troponin-tropomyosin on the binding of myosin subfragment 1 to actin. *Biochemistry.* 22:2770–2774.