Rotational Dynamics of Actin-Bound Intermediates of the Myosin Adenosine Triphosphatase Cycle in Myofibrils

Christopher L. Berger and David D. Thomas
Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455 USA

ABSTRACT We have used saturation transfer electron paramagnetic resonance (ST-EPR) to measure the microsecond rotational motion of actin-bound myosin heads in spin-labeled myofibrils in the presence of the ATP analogs AMPPNP (5′-adenylylimido-diphosphate) and ATPγS (adenosine-5′-*O*-(3-thiotriphosphate)). AMPPNP and ATPγS are believed to trap myosin in two major conformational intermediates of the actomyosin ATPase cycle, respectively known as the weakly bound and strongly bound states. Previous ST-EPR experiments with solutions of acto-S1 have demonstrated that actin-bound myosin heads are rotationally mobile on the microsecond time scale in the presence of ATPγS, but not in the presence of AMPPNP. However, it is not clear that results obtained with acto-S1 in solution can be extended to actomyosin constrained within the myofibrillar lattice. Therefore, ST-EPR spectra of spin-labeled myofibrils were analyzed explicitly in terms of the actin-bound component of myosin heads in the presence of AMPPNP and ATPγS. The fraction of actin-attached myosin heads was determined biochemically in the spin-labeled myofibrils, using the proteolytic rates actomyosin binding assay. At physiological ionic strength (μ = 165 mM), actin-bound myosin heads were found to be rotationally mobile on the microsecond time scale (τ_r = 24 ± 8 μs) in the presence of ATPγS, but not AMPPNP. Similar results were obtained at low ionic strength, confirming the acto-S1 solution studies. The microsecond rotational motions of actin-attached myosin heads in the presence of ATPγS are similar to those observed for spin-labeled myosin heads during the steady-state cycling of the actomyosin ATPase, both in solution and in an active isometric muscle fiber. These results indicate that weakly bound myosin heads, in the pre-force phase of the ATPase cycle, are rotationally mobile, while strongly bound heads, in the force-generating phase, are rotationally immobile. We propose that force generation involves a transition from a dynamically disordered crossbridge to a rigid and stereospecific one.

INTRODUCTION

Mechanochemical energy transduction in muscle contraction involves repeated cycles of attachment and detachment between the two major myofibrillar proteins, actin and myosin (Huxley, 1969; Huxley and Simmons, 1971; Huxley, 1974; Huxley and Kress, 1985). It has been proposed that the chemical energy of ATP hydrolysis by the myosin ATPase is converted to mechanical work and force generation via conformational changes of myosin while attached to actin, mediated by specific intermediate states of the actomyosin ATPase cycle (Lymn and Taylor, 1971; Eisenberg and Hill, 1985). Thus, to understand the mechanism of muscle contraction at the molecular level, it is important to understand the correlation between the biochemical kinetics and molecular dynamics of the actomyosin system. Spectroscopic probes have provided a very powerful technique for examining molecular dynamics in assemblies of macromolecular complexes such as actomyosin (reviewed by Thomas, 1987). Not only do spectroscopic experiments provide dynamic information not available from static structural techniques such as electron microscopy and x-ray diffraction, but specific sites within the macromolecular complexes can be probed with the proper choice of extrinsic label and reaction conditions. One such technique that is sensitive to the large-scale microsecond rotational motions expected of proteins such as actin and myosin during muscle contraction is saturation-transfer electron paramagnetic resonance (ST-EPR) (Thomas et al., 1976; Squier and Thomas, 1986).

Nitroxide spin labels have been attached to Cys-707 (SH1) of the myosin heavy chain to examine the rotational dynamics of the myosin head as it interacts with actin during the contractile cycle. ST-EPR, in conjunction with conventional EPR experiments, has demonstrated that most (>80%) of the myosin heads in an isometrically contracting muscle fiber are highly disordered and mobile on the microsecond time scale (Barnett and Thomas, 1989; Fajer et al., 1990). However, since the observed spectroscopic signals contain contributions from both the actin-attached and detached populations of myosin heads, information about the fractional contribution of each population is required to investigate the rotational dynamics of the actin-attached myosin heads during contraction. While stiffness measurements have been used to provide estimates of the number of actively actin-attached myosin molecules in the muscle fiber, there is not always a linear relationship between actin attachment and stiffness (Fajer et al., 1988; Pate and Cooke, 1988). Thus, it has not yet been possible to monitor unambiguously the microsecond rotational dynamics of actin-attached myosin heads in active muscle fibers. Solution studies involving purified myosin

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Address reprint requests to Dr. David D. Thomas, Department of Biochemistry, Millard 4-225, University of Minnesota Medical School, Minneapolis, MN 55455. Tel.: 612 625 0957; Fax: 612 624 0632; E-mail: ddt@ddt.biochem.umn.edu.

Dr. Berger's current address: Department of Physiology and Biophysics, University of Vermont, Burlington, VT 05405.

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subfragment-1 (S1) and actin offer the advantage that the fraction of myosin heads can be directly determined by centrifugation binding assays (Chalovich and Eisenberg, 1982). Experiments utilizing direct binding assays, in conjunction with ST-EPR on acto-S1 in solution, have previously demonstrated that actin-attached myosin heads are rotationally mobile on the microsecond time scale during the steady-state ATPase cycle (Berger et al., 1989). Most solution studies, however, must be done under nonphysiological conditions; S1 interacts significantly with actin at only very low ionic strength and is no longer mechanically constrained when attached to actin as it is within the myofibrillar lattice.

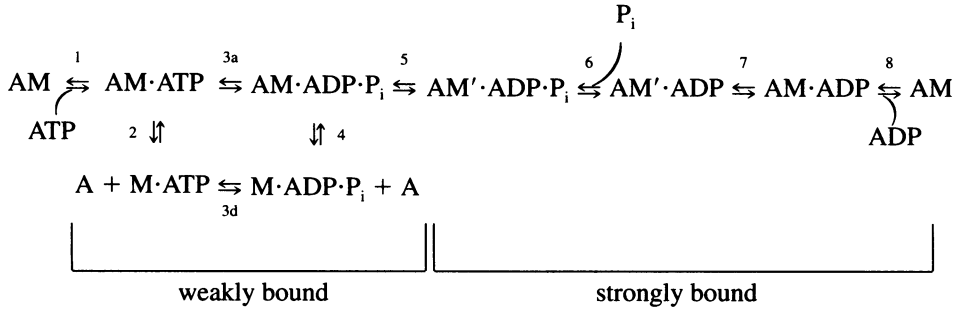
Myofibrils provide an excellent model of the intact muscle fiber in solution in which the fraction of actin-attached myosin heads can be determined. Actin and myosin are present intact in the myofibrillar lattice, constraining the two contractile proteins mechanically, and allowing them to interact even at physiological ionic strengths. Kinetic studies have shown that the steady-state ATPase activity of myofibrils is comparable to that in single muscle fibers (Glyn and Sleep, 1985), and the rate of labeled phosphate exchange is similar to that observed for isometric muscle fibers, but not solutions of acto-S1, indicating that the myosin heads are subject to mechanical strain comparable to that in the muscle fiber (Bowater and Sleep, 1985). The fraction of actin-attached myosin heads in myofibrils can be determined by monitoring the rate of tryptic digestion of the myosin heavy chain between the 50- and 20-kDa domains of the myosin head (Duong and Reisler, 1989). This site is protected when the myosin head is attached to actin, but not when it is detached (Lovell and Harrington, 1981). It is necessary to follow the initial rate of tryptic digestion of the myosin head, since the myosin heads are often in a rapid equilibrium of actin-attached and detached myosin heads during the actomyosin ATPase cycle (Duong and Reisler, 1987a,b, 1989). A limitation in determining the fraction of actin-attached myosin heads in active myofibrils is that the myofibrils must be partially cross-linked with EDC to prevent their shortening (Duong and Reisler, 1989). However, the fraction of actin-attached myosin heads in uncross-linked myofibrils can be determined in the presence of ATP analogs that are thought to trap myosin heads in specific intermediate states of the actomyosin cycle (Chen and Reisler, 1984; Azarcon et al., 1985).

Therefore, we have used the ATP analogs 5′-adenylyl-imido-diphosphate (AMPPNP) and adenosine-5′-*O*-(3-thiotriphosphate (ATPγS) to trap myosin in states that are thought to correspond to the two major conformational intermediates of the actomyosin ATPase cycle. These two major conformational states of myosin, which have been identified both through structural and kinetic means (reviewed by Eisenberg and Hill, 1985; Brenner, 1987), are often referred to as the weakly bound and strongly bound intermediates of the actomyosin ATPase cycle (Scheme 1). In the presence of AMPPNP, a nonhydrolyzable analog of ATP (Yount et al., 1971a,b), muscle fibers exhibit structural (Barrington-Leigh et al., 1972; Goody et al., 1975; Lymn, 1975; Padron and Huxley, 1984; Fajer et al., 1988) and mechanical (Marston et al., 1976; Kuhn, 1978a,b) properties intermediate between those of rigor and relaxation, and myosin is believed to be trapped in a strongly bound intermediate state of the ATPase cycle (Greene and Eisenberg, 1978). ATPγS, an analog of ATP that is hydrolyzed 500 times more slowly than ATP (Bagshaw et al., 1972; Barrington-Leigh et al., 1972), is thought to accumulate myosin in a weakly bound, prehydrolysis intermediate state (M·ATP in Scheme 1) of the ATPase cycle (Goody and Hofmann, 1980). We have previously examined the microsecond rotational dynamics of spin-labeled acto-S1 in solution in the presence of AMPPNP and ATPγS (Berger and Thomas, 1991). It was determined that myosin heads attached to actin in solution at low ionic strength are rotationally mobile on the microsecond time scale in the presence of ATPγS, but not in the presence of AMPPNP. In the present study, we have used ST-EPR and the actomyosin tryptic digestion binding assay to examine directly the rotational dynamics of spin-labeled myofibrils in the presence of ATPγS and AMPPNP. Thus, we have been able to investigate the microsecond rotational motions of two intermediate conformational states of the actomyosin ATPase cycle for actin-attached myosin heads under physiological conditions.

MATERIALS AND METHODS

Preparations and solutions

4-Maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy (MSL) was obtained from Aldrich. Dithiothreitol (DTT) and the tetralithium salts of AMPPNP and ATPγS were obtained from Boehringer-Mannheim and purified as pre-



SCHEME 1

viously described (Berger and Thomas, 1991). ATP (adenosine triphosphate), ADP (adenosine diphosphate), trypsin, soybean trypsin inhibitor, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium vanadate (Na_3VO_4) was obtained from Fisher Scientific and prepared as described previously (Barnett and Thomas, 1987). All other chemicals were of reagent grade and of the highest quality available. EPR spectroscopy and tryptic digestion were carried out in identical experimental solutions (pH 7.0, 25°C), usually containing 25 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM $CaCl_2$, and either 5 mM MgATPγS or 16 mM MgAMPPNP. In rigor solution, nucleotide was omitted. In relaxation solution, $CaCl_2$ was omitted and either 5 mM MgADP + 5 mM vanadate + 0.1 mM MgATP or 5 mM MgATPγS was added. Vanadate solutions were prepared as previously described (Goodno, 1979; Barnett and Thomas, 1987). The desired ionic strengths were achieved by the addition of the appropriate amounts of potassium propionate (KPr). The minimum ionic strength, obtained with no addition of KPr, was 45 mM in the presence of 5 mM ATPγS, and 100 mM in the presence of 16 mM AMPPNP.

Myofibrils were prepared from MSL-labeled and unlabeled glycerinated rabbit skeletal muscle (psoas) fibers as described previously (Ludescher and Thomas, 1988). Myofibrils were stored in a low ionic strength rigor buffer (25 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.0) and were always used within 2 days of preparation. The glycerinated muscle fibers were spin-labeled as described previously with the following modification: Fibers were not treated with $K_3Fe(CN)_6$, but were pretreated with the reversible sulfhydryl reagent DTNB in order block cysteines other than Cys-707 (SH1) from reacting with MSL during the labeling procedure. Briefly, fibers were incubated in rigor buffer (130 mM KPr, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 20 mM MOPS, pH 7.0) with 60 μM DTNB, conditions in which SH1 is protected. After 60 min, the excess DTNB was removed by three washes with rigor buffer. Fibers were relaxed in rigor buffer + 8 mM MgPPi, pH 6.5 to expose SH1, and MSL was added to a final concentration of 25 mM. After 20 min, MSL was removed with three washes of rigor buffer + 4 mM MgPPi, and then three washes of rigor buffer. DTNB was removed from the muscle fibers by incubation with 10 mM DTT in rigor buffer, followed by three washes of rigor buffer. The extent of labeling at SH1 and/or SH2 in the MSL myofibrils was determined to be 0.91 ± 0.03 from fractional inhibition of the high salt K^+-EDTA ATPase activity (Thomas et al., 1980), and the overall labeling specificity at SH1 and/or SH2 was determined to be 0.76 ± 0.06 from the double integration of the conventional EPR spectrum of the MSL myofibrils (Thomas et al., 1980). The concentration of protein in the myofibril preparations was determined using the biuret assay.

Tryptic digestion: determine fraction of actin-bound heads

To determine the fraction of actin-bound myosin heads within myofibrils under EPR conditions, tryptic digestion experiments were done by the method of Duong and Reisler (1989). These measurements take advantage of the three principal tryptic cleavage sites within the myosin heavy chain; between the 25- and 50-kDa fragments of S1 (T_1), between the 50- and 20-kDa fragments of S1 (T_2), and in the hinge region of the myosin rod (T_3). While the rates of tryptic digestion at T_1 (fast) and T_3 (slow) are relatively constant, the rate of digestion at T_2 is quite dependent on the attachment state of the myosin head. When detached from actin, the myosin head is highly susceptible to tryptic cleavage at T_2, but this site is protected from proteolytic attack when the myosin head is bound to actin. The major products of the myosin heavy chain in the early stages of tryptic digestion are the intact 225-kDa heavy chain, the 200-kDa product cleaved at T_1, and the 150-kDa product cleaved at T_2. In a mixture of actin-bound and free heads, the fraction of bound myosin heads (f_B) could, in principle, be determined from the relative proportions of these three digestion products. However, since the crossbridges in the presence of AMPPNP and ATPγS are in a rapid equilibrium between the attached and detached states (Schoenberg and Eisenberg, 1985; Schoenberg, 1988), all of the myosin heads will eventually be cleaved at T_2. Therefore, it is necessary to measure the initial rate k of tryptic digestion at T_2:

$$k = f_B k_B + (1 - f_B) k_F \quad (1)$$

where k_B and k_F are the rates of digestion at T_2 for actin-bound and free myosin heads (measured in separate control experiments), and k is the observed rate for an equilibrium mixture of bound (fraction f_B) and free ($1 - f_B$) heads. Therefore, the fraction of bound heads can be calculated from

$$f_B = \frac{k_F - k}{k_F - k_B} \quad (2)$$

This expression assumes that the rates of association and dissociation between actin and myosin are much faster than k_B and k_F, so that an equilibrium population of the myosin heads is sampled. This assumption is easily satisfied under the conditions of this assay (Duong and Reisler, 1989).

MSL myofibrils were adjusted to 0.5 mg/ml and digested with 0.01 mg/ml trypsin for 5 min in the same buffers used for EPR spectroscopy. Digestion mixtures were maintained at 25°C in a thermostatically controlled water bath with constant stirring. The digestion reaction was started with the addition of nucleotide, which was added immediately after the trypsin. Small aliquots were removed from the digestion mixture before the addition of trypsin, and at 1-min time intervals after the start of the reaction, and quenched in an equal volume of 0.06 mg/ml soybean trypsin inhibitor. The intensities of the 225-, 200-, and 150-kDa bands of the myosin heavy chain, as well as the 42-kDa actin band, were determined by scanning densitometry for each sample after it had been run on SDS/PAGE. The decrease in intensity of the myosin heavy chain 225- and 220-kDa bands was monitored as a function of time, and the resulting decays could be fit to a single exponential describing the rate of tryptic digestion. SDS/PAGE was done by the method of Laemmli (1970) using 7.5% (w/w) polyacrylamide gels. Optical densities of the Coomassie blue stained protein bands were determined using a Hoefer GS-300 scanning densitometer interfaced with Hoefer GS-365 scanning densitometry software on an IBM-compatible computer. All bands were normalized to their respective molecular weights to correct for differences in dye absorption, and the intensity of all myosin bands were normalized to the intensity of the actin band to account for any variations in gel loading between samples.

The fraction of myosin heads bound to actin (f_B) in MSL myofibrils was then determined from Eq. 2; k_B was determined from the digestion of rigor MSL myofibrils, k_F was determined from the digestion of MSL myofibrils relaxed at physiological ionic strength (μ = 165 mM) with 5 mM MgADP, 5 mM vanadate, and 0.1 mM MgATP in the absence of Ca^{2+}, and k was determined from the tryptic digestion of myofibrils in the same buffer conditions used during EPR spectroscopy. Normal relaxing conditions (5 mM MgATP, no Ca^{2+}) were not sufficient to measure k_F, since the thin filaments become activated due to the tryptic degradation of troponin C, but the inclusion of vanadate results in the dissociation of all heads from actin, even during tryptic digestion (Duong and Reisler, 1989).

EPR spectroscopy

EPR and ST-EPR spectra were obtained using a Bruker ESP 300 spectrometer equipped with a TE_{102} cavity. Conventional EPR (V_1) spectra were obtained using 100-kHz field modulation (with a peak-to-peak modulation amplitude of 2 G), with a microwave field intensity (H_1) of 0.032 G. ST-EPR (V'_2) spectra were obtained using 50-kHz field modulation (with a peak-to-peak modulation amplitude of 5 G), with a microwave field intensity (H_1) of 0.25 G. Spectral baselines were usually 100 G wide. The temperature was maintained at 25°C by flowing precooled N_2 over the sample, which was regulated by a variable temperature controller (Bruker). Samples were contained in a fused silica tissue cell with TPX coverplate (Wilmad Glass Co.). MSL myofibril ST-EPR samples typically contained 20–30 mg of total protein, corresponding to ~40–60 μM MSL-labeled myosin heads. Digitized EPR spectra (1024 points/scan) were acquired with the ESP 300 spectrometer's built-in Bruker OS-9-compatible ESP 1620 spectral acquisition software, and were then transferred to an IBM-compatible microcomputer. All ST-EPR spectra were analyzed using a program developed by Robert L. H. Bennett. Four to sixteen 200-s scans were typically averaged together. All ST-EPR spectra (V'_2) were normalized by dividing by the double integral of the low power (H_1 = 0.032 G) conventional EPR spectrum (V_1),

a parameter that is independent of rotational motion and corrects for any variation in the concentration of spin labels between samples (Squier and Thomas, 1986). Effective rotational correlation times (τ_r) were determined for steady-state ST-EPR spectra using calibration curves of the line-height ratio parameter C'/C, where C is the major peak in the center of the spectrum and C' is the trough immediately following C (Thomas, et al., 1976; Squier and Thomas, 1986).

Rotational motion of ternary complex

Within the myofibril, myosin can exist in four different states: myosin alone (M), actomyosin (AM), the myosin-nucleotide complex (MN), or the actomyosin-nucleotide ternary complex (AMN) (Scheme 2). The primary

$$\begin{array}{ccc} & K_4[N] & \\ \mathrm{AM} & \rightleftarrows & \mathrm{AMN} \\ K_2[A] \uparrow\downarrow & & \uparrow\downarrow K_3[A] \\ \mathrm{M} & \leftrightarrows & \mathrm{MN} \\ & K_1[N] & \end{array}$$

Scheme 2

goal of the present study is to measure the rotational motion of myosin heads that are in the ternary complex AMN, where N is either AMPPNP or ATPγS. However, the ST-EPR spectrum S of acto-S1 or myofibrils in the presence of nucleotide will be a composite of the spectra of each of these states (S_M, S_{AM}, S_{MN}, S_{AMN}), weighted by their respective mole fractions (f_N, f_{AM}, f_{MN}, f_{AMN}):

$$S = f_M S_M + f_{AM} S_{AM} + f_{MN} S_{MN} + f_{AMN} S_{AMN} \quad (3)$$

The first term is negligible, since all myosin heads in the myofibril bind to actin in the absence of nucleotide (Thomas and Cooke, 1980). The desired spectrum can be calculated by rearranging the remaining terms of Eq. 3:

$$S_{AMN} = \frac{S - f_{MN} S_{MN} - f_{AM} S_{AM}}{f_{AMN}} \quad (4)$$

All of the terms on the right of Eq. 4 can be determined experimentally. The spectra S_{MN} and S_{AM} are recorded in relaxation and rigor, respectively. The mole fraction f_{MN} is the fraction of free heads ($1 - f_B$), which is determined from tryptic digestion using Eq. 2. Finally, according to Scheme 2,

$$f_{AM} = \frac{f_B}{1 + K_4[N]}, \quad f_{AMN} = f_B - f_{AM} \quad (5)$$

It has been demonstrated previously that K_4 for various nucleotides is the same in acto-S1, myofibrils, and muscle fibers (Johnson and Adams, 1984; Biosca et al., 1988; Fajer et al., 1988). Thus assuming that the value of K_4 determined for ATPγS binding to acto-MSL-S1 in solution (>10^4 M^{-1}, Berger and Thomas, 1991) is valid in myofibrils, 5 mM MgATPγS is sufficient to saturate the actomyosin nucleotide binding sites in myofibrils, i.e., f_{AM} (Eq. 5) is negligible, so $f_{AMN} = f_B$. However, K_4 is only 320 M^{-1} for AMPPNP (Berger and Thomas, 1991), so a small fraction (16%) of myosin heads remain in rigor in the presence of 16 mM MgAMPPNP, due to its weaker affinity for actomyosin.

RESULTS

Tryptic digestion measurements of crossbridge binding

MSL myofibrils were digested with trypsin to determine the fraction of myosin heads bound to actin in the presence of ATPγS and AMPPNP, using the proteolytic rates method (Duong and Reisler, 1989). Typical data are shown in Fig. 1, and the results are summarized and quantitated in Table 1. No digestion was detectable in rigor (so $k_B = 0$, Fig. 1), either at low or physiological ionic strength, indicating that all the myosin heads were bound to actin. The rate of digestion in relaxation was $k_F = 3.21 \pm 0.19 \times 10^{-3}$ s^{-1} (Fig. 1). At physiological ionic strength, the digestion rate was $0.90 \pm 0.06 \times 10^{-3}$ s^{-1} in the presence of 16 mM MgAMPPNP (Fig. 1), corresponding to 0.72 ± 0.02 of the myosin heads being bound to actin; 0.16 in a rigor complex and 0.56 in a ternary complex with actin and MgAMPPNP. In the presence of 5 mM MgATPγS at physiological ionic strength, the digestion rate was $2.72 \pm 0.08 \times 10^{-3}$ s^{-1} (Fig. 1), corresponding to 0.15 ± 0.03 of the myosin heads being bound to actin, all of which are in a ternary complex with ATPγS. At low ionic strength, the digestion rates were $0.49 \pm 0.09 \times 10^{-3}$ s^{-1} in the presence of 16 mM MgAMPPNP and $2.18 \pm 0.11 \times 10^{-3}$ s^{-1} in the presence of 5 mM MgATPγS, corresponding to 0.85 ± 0.03 and 0.32 ± 04 of the myosin heads being bound to actin, respectively. Only 0.69 of the actin-bound myosin heads were in the ternary complex with MgAMPPNP due to the small fraction of rigor-like crossbridges (0.16), but all of the actin-bound myosin heads were in a ternary complex with MgATPγS. The rates of tryptic digestion were also measured for unlabeled myofibrils at physiological ionic strength, and these values were not significantly different from those measured for MSL myofibrils (data not shown).

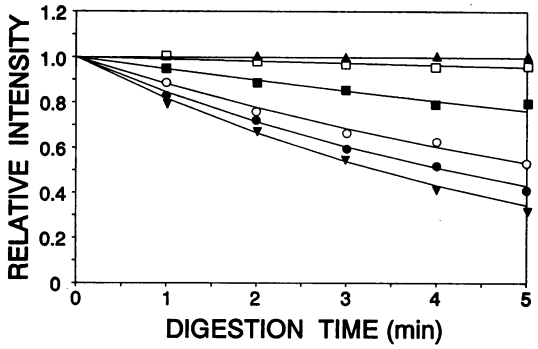


FIGURE 1 Rate of tryptic digestion of MSL myofibrils. The intensity of the myosin heavy chain (225- and 200-kDa bands) is plotted as a function of tryptic digestion time. Values have been normalized to the initial intact heavy chain intensity. Rates of tryptic digestion were determined from a single exponential fit to the data, shown as a solid line. Incubations were performed at 25°C and contained 0.5 mg/ml EDC-MSL myofibrils and 0.01 mg/ml trypsin. (▼) relaxed at physiological ionic strength (μ = 165 mM) with 3 mM MgADP.Vi + 0.1 mM MgATP. (▲) Rigor physiological ionic strength (μ = 165 mM). (□) 16 mM MgAMPPNP at low ionic strength (μ = 100 mM). (■) 16 mM MgAMPPNP at physiological ionic strength (μ = 165 mM). (○) 5 mM MgATPγS at low ionic strength (μ = 45 mM). (●) 5 mM MgATPγS at physiological ionic strength (μ = 165 mM).

ST-EPR experiments

In the absence of nucleotide, the high ST-EPR spectral intensity of MSL myofibrils indicates that all of the myosin heads are rigidly bound to actin on the microsecond time scale (Fig. 2), regardless of ionic strength. Due to the rather long time period (20–30 min) required to acquire a full ST-EPR spectrum, and the high concentration of myofibrils (20–30 mg/ml) required for adequate signal intensity, MSL myofibrils were relaxed at physiological ionic strength

TABLE 1 Results of tryptic digestion and ST-EPR experiments*

	IS (mM)	$k \times 10^{-3}$ (s^{-1})	f_B	C'/C	τ_r (μs)
Controls					
Rigor	165	0.00 ± 0.03	≡ 1	0.74 ± 0.08	100 ± 9
Relaxed	165	3.21 ± 0.19	≡ 0	−0.25 ± 0.06	1.1 ± 0.2
Composite data					
MgAMPPNP	165	0.90 ± 0.06	0.72 ± 0.02	0.47 ± 0.09	42 ± 13
MgAMPPNP	100	0.49 ± 0.09	0.85 ± 0.03	0.63 ± 0.07	96 ± 7
MgATPγS	165	2.72 ± 0.08	0.15 ± 0.03	−0.22 ± 0.06	4.1 ± 0.8
MgATPγS	45	2.18 ± 0.11	0.32 ± 0.04	−0.06 ± 0.04	5.4 ± 0.4
Actin-bound data					
MgAMPPNP	165		≡ 1	0.72 ± 0.14	99 ± 13
MgAMPPNP	100		≡ 1	0.71 ± 0.09	97 ± 7
MgATPγS	165		≡ 1	0.32 ± 0.11	24 ± 8
MgATPγS	45		≡ 1	0.33 ± 0.08	24 ± 5

* IS is ionic strength. k is the rate of tryptic digestion, which is used to determine f_B, the fraction of myosin heads bound to actin (Eq. 1). C'/C is the line-height ratio parameter used to determine τ_r, the effective rotational correlation time. EPR spectra of actin-bound heads were obtained using Eq. 4, as illustrated in Figs. 3 and 4. All errors are given as SEM, n = 4–5.

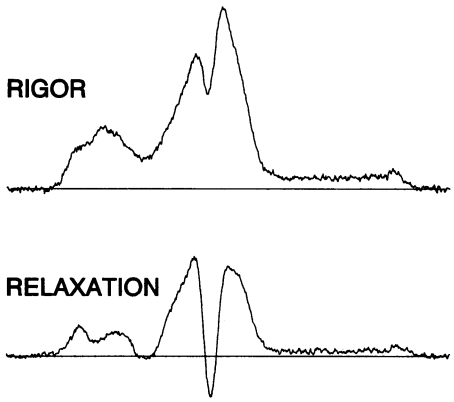


FIGURE 2 ST-EPR spectra of MSL myofibrils at physiological ionic strength (μ = 165 mM) relaxed with 5 mM MgADP + 5 mM vanadate + 0.1 mM MgATP (*bottom*) or in the absence of nucleotide (rigor, *top*). The protein concentration was 20–30 mg/ml. Each spectral baseline is 100 G wide.

(μ = 165 mM) in the presence of 3 mM MgADP.Vi and 0.1 mM MgATP or in the presence of 5 mM MgATPγS. There was no significant difference in the ST-EPR spectra of the MSL myofibrils relaxed by either method (data not shown), demonstrating that 5 mM MgATPγS was sufficient to saturate the myosin active sites. The ST-EPR spectral intensity of relaxed MSL myofibrils was quite low relative to the rigor spectrum (Fig. 2), indicating considerable microsecond rotational motion of the myosin heads. Effective rotational correlation times (τ_r), derived from calibration curves of spin-labeled hemoglobin with known values of τ_r (Squier and Thomas, 1986), for the myosin heads under conditions of rigor and relaxation, were determined to be 100 ± 9 μs and 1.1 ± 0.2 μs, respectively. The observed microsecond rotational motions of myosin heads in a rigor complex with actin have been shown to arise from flexibility within the actin filament (Thomas et al., 1980; Ostap and Thomas, 1991).

At low (μ = 100 mM) and physiological (μ = 165) ionic strength, the rotational motion of the MSL myofibrils in the presence of 16 mM MgAMPPNP is intermediate between those of rigor myofibrils and relaxed myofibrils (Fig. 3). At the lower ionic strength, the myosin heads in MSL myofibrils have only slightly more microsecond rotational motion in the presence of 16 mM MgAMPPNP than rigor crossbridges, with a τ_r value of 96 ± 7 μs. Increasing the ionic strength to physiological levels only increases the rotational motion in MSL myofibrils in the presence of 16 mM MgAMPPNP by a factor of two (τ_r = 42 ± 13 μs). A ST-EPR spectrum of the actin-bound myosin heads in the ternary complex with AMPPNP in MSL myofibrils (Fig. 3) was obtained at both low and physiological ionic strength after correction for the fraction of myosin heads detached from actin (0.28 at μ = 165 mM, 0.05 at μ = 100 mM) and in rigor (assumed to be 0.16 at both low and physiological ionic strength). The value of τ_r (97 ± 7 μs at μ = 100 mM and 99 ± 13 μs at μ = 165 mM) determined for the actomyosin-AMPPNP ternary complex in MSL myofibrils was independent of ionic strength, and indicated no rotational motion on the microsecond time scale. Thus myosin heads bound to actin in the ternary complex with AMPPNP in the myofibrillar lattice are rigidly attached, just as in rigor.

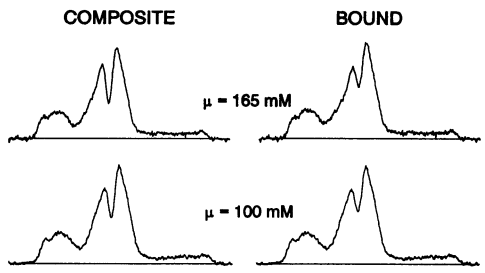


FIGURE 3 ST-EPR spectra of MSL myofibrils (*left*) and the actin-bound myosin heads (*right*) in the presence of 16 mM MgAMPPNP and 1 mM free Ca^{2+} at physiological (μ = 165 mM, first row) and low (μ = 100 mM, second row) ionic strength, at 25°C. The spectrum of the actin-bound myosin heads (*right*) was obtained by subtracting the appropriate mole fractions of the relaxed MSL myofibril (corresponding to the detached myosin heads) and rigor MSL myofibril spectra from the composite spectrum (Eq. 4). The protein concentration was 20–30 mg/ml. Each spectral baseline is 100 G wide.

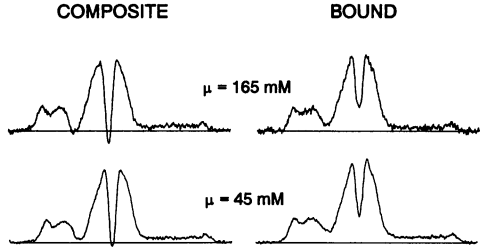


FIGURE 4 ST-EPR spectra of MSL myofibrils (*left*) and the actin-bound myosin heads (*right*) in the presence of 5 mM MgATPγS and 1 mM free Ca^{2+} at physiological (μ = 165 mM, first row) and low (μ = 45 mM, second row) ionic strength, at 25°C. The spectrum of the actin-bound myosin heads (*right*) was obtained by subtracting the appropriate mole fraction of the relaxed MSL myofibril (corresponding to the detached myosin heads) spectrum from the composite spectrum (Eq. 5). The protein concentration was 20–30 mg/ml. Each spectral baseline is 100 G wide.

In the presence of 5 mM MgATPγS, myosin heads in MSL myofibrils have almost as much microsecond rotational motion as those in relaxed MSL myofibrils (Fig. 4). At physiological ionic strength (μ = 165 mM) in the presence of 5 mM MgATPγS, the myosin heads are rotating on the microsecond time scale with a τ_r value of 4.1 ± 0.8 μs, and despite a significant difference (20%) in the fraction of actin-bound myosin heads, the microsecond rotational motion of MSL myofibrils in the presence of 5 mM MgATPγS at low ionic strength (μ = 45 mM) increases only slightly, with a τ_r of 5.4 ± 0.4 μs. The ST-EPR spectrum of the actomyosin-ATPγS ternary complex was obtained for the MSL myofibrils at both low and physiological ionic strength after correction for the fraction of dissociated myosin heads (0.85 at physiological ionic strength and 0.68 at low ionic strength). It was not necessary to correct the spectra for a fraction of rigor heads as in the presence of AMPPNP, since 5 mM MgATPγS was sufficient to saturate all of the myosin nucleotide binding sites in MSL myofibrils. The actin-bound myosin heads in the presence of 5 mM MgATPγS were independent of ionic strength in MSL myofibrils, and are rotationally mobile on the microsecond time scale (Fig. 4), with τ_r values of 24 ± 5 μs at low ionic strength and 24 ± 8 μs at physiological ionic strength. Thus, in contrast to the rigidly-attached myosin heads we observed in the presence of AMPPNP, myosin heads bound to actin in the presence of ATPγS undergo large-scale microsecond rotational motions within the myofibrillar lattice.

DISCUSSION

Interpretation of tryptic digestion rates

Determining the fraction of actin-bound myosin heads in MSL myofibrils depends on the rate of tryptic digestion at T_2 of the detached myosin heads, measured in relaxed myofibrils, compared with actin-bound myosin heads measured in rigor myofibrils (Eq. 1). Thus it was important to ensure that all of the myosin heads in the relaxed myofibrils were truly detached, since the thin filament can become activated by degradation of troponin C during the tryptic digestion binding experiments (Duong and Reisler, 1989). Myofibrils under these conditions are digested by trypsin at the same rate as *p*-nitrophenylenemaleimide (pNPM) modified myofibrils, in which the actomyosin affinity in the presence of ATP has been shown to be negligible (Duong and Reisler, 1989), and the active tension and stiffness of isometric muscle fibers fall to 0 under these conditions (Goody et al., 1980). The inclusion of a small amount of ATP with ADP.V_i is necessary for relaxation, since vanadate binds to a crossbridge state that is only accessible during the active ATPase cycle (Dantzig and Goldman, 1984). In agreement with previous results (Lovell and Harrington, 1981; Chen and Reisler, 1984; Duong and Reisler, 1989), we found that myosin heads are well protected from tryptic digestion at T_2 in a rigor complex with actin. However, a key assumption in these experiments is that the rate of tryptic digestion of actin-bound myosin heads is the same in the presence and absence of nucleotide. It has been demonstrated that the fraction of actin-bound myosin heads in solutions of acto-S1 in the presence of ATP (Duong and Reisler, 1987a), determined from the rate of tryptic digestion, agrees exactly with sedimentation binding measurements under the same conditions. Therefore, rigor crossbridges appear to be a satisfactory model for the proteolytic susceptibility of all actin-attached myosin heads, whether nucleotide is present or not. Another implicit assumption is that the equilibrium population of actin-attached and detached crossbridges be sampled. The rate of tryptic digestion in both rigor and relaxation was several orders of magnitude slower than the association and dissociation between actin and myosin under these conditions (Schoenberg, 1988), so Eqs. 1 and 2 are valid.

The fraction of myosin heads bound to actin in myofibrils in the presence of AMPPNP agrees closely with values measured previously in both cardiac (Azarcon et al., 1985) and skeletal (Chen and Reisler, 1984) myofibrils at physiological (0.72) and low (0.85) ionic strengths. These values do not take into account the fraction of rigor heads (AM) present in the myofibrils due to the weak affinity of AMPPNP for actomyosin. It is much more difficult to determine the fraction of actin-bound myosin heads in the ternary complex with AMPPNP from the myofibril binding experiments than from the acto-S1 binding experiments, due to the uncertainties of the actin and myosin concentrations in the myofibril. However, it has been demonstrated that the affinity of actin-bound S1 for AMPPNP in solution is similar to that in the muscle fiber, as determined by competitive inhibition with spin-labeled ADP (Fajer et al., 1988), and in myofibrils, as determined by the amount of bound radioactively labeled nucleotide (Johnson and Adams, 1984; Biosca et al., 1988). This supports our assumption that the value of K_4 measured for AMPPNP and ATPγS with solutions of acto-S1 is applicable to the myofibril experiments.

The binding of myosin heads to actin in myofibrils has not been previously measured in the presence of ATPγS. Based on our previous direct measurements of acto-S1 binding

(Berger and Thomas, 1991), the affinity of ATPγS is sufficiently strong ($K_4 > 10^4\ M^{-1}$) to saturate completely the myosin nucleotide binding sites in myofibrils under the conditions of this study (5 mM ATPγS), both in the presence and absence of Ca^{2+}. In the absence of Ca^{2+}, saturation by 5 mM ATPγS is clearly supported by our observation that the ST-EPR spectrum is the same as in relaxation (Fig. 4, Table 1), by previous results of fiber stiffness (Dantzig et al., 1988) and x-ray diffraction (Barrington-Leigh et al., 1972), and by competition with spin-labeled ATP (P. Fajer, personal communication). In the presence of Ca^{2+}, mechanical measurements support our conclusion that 5 mM ATPγS saturates (Dantzig et al., 1988; Kraft et al., 1992). x-ray diffraction changes in the presence of Ca^{2+} have been observed at ATPγS concentrations above 5 mM (Kraft et al., 1992); in light of the more direct and myosin-specific measurements of binding and mechanics cited above, these x-ray changes are probably due to nonspecific binding at low-affinity sites other than the myosin active site. Our binding results are consistent with mechanical results comparing ATPγS fibers with active fibers: in the presence of saturating ATPγS and Ca^{2+}, muscle fiber stiffness (in response to rapid stretch) is ~50% of the active level (Dantzig et al., 1988). Similarly, the fraction of actin-attached myosin heads in the presence of ATPγS (0.15 ± 0.03, Table 1) is close to half of the value obtained in the presence of ATP (0.24 ± 0.04) at physiological ionic strength in myofibrils (Berger and Thomas, 1993).

Interpretation of ST-EPR results in the presence of AMPPNP

Myosin heads in the presence of AMPPNP are rigidly bound to actin in MSL myofibrils, independent of ionic strength, just as in the absence of nucleotide (rigor), with a τ_r of ~100 μs. The submillisecond motions that are observed are due to the flexibility of the actin filaments to which the myosin heads are bound (Thomas et al., 1979, 1980; Ostap and Thomas, 1991). These results are consistent with a previous study of AMPPNP effects on the orientation and rotational motion of MSL-labeled myosin heads in muscle fibers (Fajer et al., 1988), in which it was shown that approximately half of the myosin heads in the presence of saturating AMPPNP are oriented and immobile on the microsecond time scale as in rigor, while the other half are dynamically disordered as in relaxation, despite no change in stiffness. These results are also consistent with experiments on acto-MSL-S1 in solution (Berger and Thomas, 1991), in which it was shown directly by ST-EPR, in conjunction with sedimentation binding measurements, that myosin heads are rigidly bound to actin in the presence of AMPPNP. ST-EPR studies of HMM (Manuck et al., 1986) and of myofibrils (Ishiwata et al., 1986) have also shown that crossbridges in the presence of AMPPNP are composed of two components, one that is rigid, and the other rotationally mobile, on the microsecond time scale. The results from the present work show that the myosin heads bound to actin in the presence of AMPPNP are the immobile population observed previously with HMM, myofibrils, and muscle fibers, and that the mobile population observed are detached from actin under these conditions.

Since actin-attached myosin heads with AMPPNP bound have the same orientation and rotational rigidity as those in rigor, or with ADP bound (Manuck et al., 1986; Fajer et al., 1988), their conformation probably represents a state late in the actomyosin ATPase cycle, in which the affinity for actin is relatively strong. Biochemical evidence supports this conclusion, and mechanical and structural studies indicate that the AMPPNP state is probably intermediate between the ADP and relaxed states. The association constants of AMPPNP binding to myosin and actomyosin are closer to those of ADP than ATP (Greene and Eisenberg, 1978, 1980), and S1 binds to regulated actin in the absence of Ca^{2+} with positive cooperativity in the presence of AMPPNP, just as in the presence of ADP, but not ATP (Greene, 1982; Williams and Greene, 1983). However, electron microscopy coordinated with x-ray diffraction of insect flight muscle (Reedy et al., 1983), x-ray diffraction of vertebrate muscle (Lymn, 1975; Padron and Huxley, 1984), and EPR of spin-labeled myosin light chains in vertebrate muscle (Arata, 1990) suggest that the distal portion of the AMPPNP-bound head is ordered with the thick filament rather than the thin filament as in the ADP and rigor states. Crossbridges in HMM (Greene, 1981; Duong and Reisler, 1987b), in myofibrils (Chen and Reisler, 1984), and in fibers (Fajer et al., 1988) are characterized by single-headed binding to actin in the presence of AMPPNP or ADP, in contrast to rigor crossbridges, which are bound to actin by both myosin heads (Greene and Eisenberg, 1980; Thomas and Cooke, 1980). These results suggest that the AMPPNP state is structurally different from the ADP or rigor states and may represent an earlier state in the ATPase cycle. Mechanical measurements on muscle fibers in the presence of AMPPNP have also suggested that this state represents a partial reversal of the power stroke (Marston et al., 1976; Kuhn, 1978a,b), although crossbridge detachment and reattachment to positions of lower strain cannot be ruled out in these experiments (Schoenberg, 1989). Thus, AMPPNP appears to trap myosin in a late intermediate state of the actomyosin ATPase cycle that is similar, but not identical, to the ADP and rigor states. The ambiguity of crossbridge attachment in the previous structural and mechanical experiments with AMPPNP make it difficult to discern effects of crossbridge detachment from changes in the actin-attached crossbridge. In the present work, we have been able to differentiate the rotational motions of the actin-attached myosin heads from those arising from the dissociation of crossbridges from actin. These results make it clear that the A.M.AMPPNP complex, an analog of strongly-bound states late in the actomyosin ATPase cycle, is characterized by heads that are rotationally immobile relative to actin on the microsecond time scale.

Interpretation of ST-EPR results in the presence of ATPγS

Myosin heads bound to actin in MSL myofibrils at physiological and low ionic strengths in the presence of ATPγS

have considerable microsecond rotational mobility (τ_r = 24 ± 6 μs), in agreement with previous results on actin.MSL-S1 in solution at low ionic strength (17 ± 2 μs; Berger and Thomas, 1991). The slightly larger τ_r (indicating slower or more restricted rotational motion) in myofibrils is probably due to the greater restriction of the myosin head when attached to the thick filament backbone of the myofibrillar lattice. The A.M.ATPγS state is more mobile than the rigor complex (τ_r = 100 μs, Table 1) but less mobile than the detached head (τ_r = 1.1 μs, relaxed control in Table 1).

The τ_r values reported in Table 1 are probably not accurate values for the time constants of rotational motion, since they are effective correlation times calculated assuming that all the bound heads undergo large-amplitude (>90°) rotational motion at the same rate (Squier et al., 1986). Since an increase in τ_r can arise from either an increase in the actual time constant of motion or a decrease in the angular amplitude (Howard et al., 1993), the reported τ_r is an upper bound for the actual time constant of motion. For example, if the actual time constant for bound heads in A.M.ATPγS is the same as for free heads (1.1 μs), the angular amplitude of motion is 23° (Gaussian full width at half-maximum, from Fig. 9 in Howard et al., 1993). Similarly, if the bound heads have more than one mobility state, the reported τ_r value is intermediate between the values for the two states. For example, if some bound A.M.ATPγS heads are as immobile as in rigor and the rest have the same motion as free heads, the fraction mobile is 0.36 (from linear interpolation of the ST-EPR spectra). Conventional EPR studies on oriented fibers will be needed to determine the number of orientational states and the amplitudes of motion. Nevertheless, from the present data we can conclude unambiguously that, for the bound A.M.ATPγS state, 1) at least 36% of the heads are mobile, 2) the time constant for the mobile heads is no greater than 24 μs, and 3) the amplitude for the mobile heads is at least 23°.

In the presence of ATPγS and Ca^{2+} at physiological or low ionic strengths, muscle fibers develop stiffness without developing tension (Dantzig et al., 1988; Kraft et al., 1992), suggesting that the myosin heads are in a weak-binding state, early in the ATPase cycle, that binds to actin but does not generate force. The dependence of stiffness on the speed of stretch suggests that these crossbridges have a wide range of detachment rates, suggesting that multiple attached states exist (Dantzig et al., 1988; Kraft et al., 1992). Transitions between multiple attached states might account for the observed microsecond rotational motions of the ternary A.M.ATPγS complex, but time-resolved experiments will be necessary to test this hypothesis. Since ATPγS is hydrolyzed by myosin ~500 times more slowly than is ATP, and the rate of product release is much slower than the hydrolysis step (Bagshaw et al., 1972; Goody and Hofmann, 1980), ATPγS probably traps myosin in an early, prehydrolysis intermediate of the actomyosin ATPase cycle, analogous to the A.M.ATP state in Scheme 1 (Bagshaw et al., 1972; Barrington-Leigh et al., 1972; Goody et al., 1975). Our results clearly demonstrate that in contrast to the later stages of the ATPase cycle, myosin heads in weakly bound states (as modeled by ATPγS) are dynamically attached to actin, even within the constraints of the myofibrillar lattice.

The existence of dynamic actin-bound myosin heads in weakly bound states of the ATPase cycle is supported by evidence from other techniques. X-ray diffraction of relaxed skeletal muscle fibers (Yu and Brenner, 1989; Harford and Squire, 1992), indicate that the actin-attached crossbridges in the presence of ATP are structurally distinct from rigor crossbridges, based on changes in the radial mass distribution around the thick and thin filaments. Most electron microscopic studies of myosin heads bound to actin in solution during the ATPase steady state, in which weak binding states predominate, have shown a high degree of angular head disorder that is quite distinct from the highly ordered rigor state (Craig et al., 1985; Applegate and Flicker, 1987; Katayama, 1989; Frado and Craig, 1992), although a recent study reported rigor-like heads (Pollard et al., 1993).

Comparison with active myofibrils

The value of τ_r (24 μs) measured for the actin-attached myosin heads in MSL myofibrils in the presence of ATPγS is similar to the value measured for actin-attached myosin heads in active MSL myofibrils (Berger and Thomas, 1993), and isometrically contracting spin-labeled (Barnett and Thomas, 1989) and phosphorescent-labeled (Stein et al., 1990) muscle fibers, assuming the level of crossbridge attachment estimated from stiffness measurements. Conventional EPR of isometrically contracting MSL-labeled muscle fibers found a small but significant (12 ± 4%) population of myosin heads to be in a rigor-like orientation, and the rest dynamically disoriented (Fajer et al., 1990). Similarly, electron micrographs of isometric muscle frozen rapidly after caged ATP photolysis show a high degree of angular disorder of (apparently) attached crossbridges even during maximum force production (Hirose et al., 1993); and x-ray diffraction patterns of active muscle are also consistent with most heads (85%) being disordered (Lowy and Poulsen, 1987). Given that the fraction of attached myosin heads is >12%, whether estimated by stiffness in muscle fibers or determined directly in myofibrils by tryptic digestion (24%, Berger and Thomas, 1993), this result suggests that actin-bound heads in active muscle are in at least two motion states: a relatively rigid, rigor-like state and a more rotationally mobile state. Based on the results in the present work, it is plausible that crossbridges in weakly bound, pre-force-generating states of the actomyosin ATPase cycle are the rotationally mobile population of actin-attached myosin heads observed in the active muscle, and the strongly bound, force-producing states are rigidly attached to actin.

The power stroke as a disorder-to-order transition

In the most commonly discussed model of muscle contraction, the power stroke is seen as a transition between two states of well-defined stereospecific myosin-actin interaction; a pre-force state in which myosin heads bind weakly to

actin at an ~90° angle, and a force-producing state in which myosin heads bind more strongly and proceed to a 45° (rigor-like) angle. However, no distinct orientation significantly different from rigor has been observed by EPR or any other technique. On the basis of the present results, and the previous results on active fibers and myofibrils discussed above, we propose that the power stroke is instead a transition from a dynamically disordered state, with little stereospecificity, to an ordered state in which the head is rigidly oriented as in rigor (Fig. 5). Although this model allows for more disorder than the conventional model, it is clearly capable of producing directional force, since the average net rotation is the same as in the 45–90° model. This proposal is consistent with a model of the actin-myosin interaction based on the high-resolution structures of actin and S1, in which specific surfaces of complementary charge on the two proteins are proposed to provide an initial weak ionic interaction, which becomes strong and stereospecific only when a force-generating structural transition produces more extensive hydrophobic interactions (Rayment et al., 1993). That model also suggested an important role for internal structural changes within the myosin head; although changes of this sort do occur, as detected by EPR (Ostap et al., 1993) and other techniques, the model in Fig. 5 does not speculate about how they might be involved.

Despite the apparent simplicity of Fig. 5, the existing data do not require that all of the weakly bound heads are mobile (as discussed above), nor is it clear that only the weakly bound heads are mobile. Further high-resolution studies correlating molecular dynamics with force production will be necessary to examine more directly the rotational dynamics of the force-producing heads.

In any case, it is likely that the weakly bound intermediates of the actomyosin ATPase cycle play an important role in the process of force generation (Huxley and Kress, 1985). For example, the actin-binding proteolytic fragment of caldesmon that specifically inhibits the formation of weakly bound crossbridges, but not strongly bound ones that can generate force, has been shown to inhibit force production in active muscle fibers at physiological ionic strength (Brenner et al., 1991). The dynamic nature of active crossbridge attachment is further underscored by the conclusion that crossbridges may dissociate and reassociate rapidly in isometric contraction (Brenner, 1991) and that an active myosin head may interact with many different actin molecules during a single ATPase cycle (Harada et al., 1990). Thus there is considerable structural and mechanical evidence that supports the existence of a rotationally dynamic actin-attached myosin head in the active crossbridge cycle. The microsecond rotational motions we have observed for actin-attached myosin heads, under physiological conditions in the myofibrillar lattice, are probably an integral part of the molecular mechanism of muscle contraction.

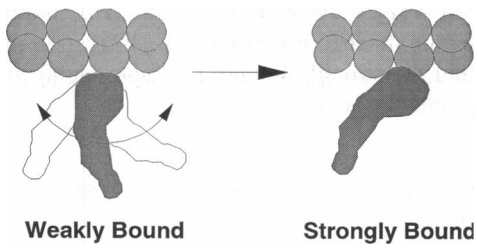


FIGURE 5 A model depicting schematically how a transition from orientational disorder, in weakly bound crossbridge states, to rigor-like order, in strongly bound states, could produce directional force and filament sliding.

Alternative explanations

We have considered the possibility that S1 remains rigidly attached to actin even in the presence of ATP and that the nucleotide induces rotational motions in actin. Experiments using spin-labeled actin (at Cys-374), rather than S1, indicated that there is no change in the rotational mobility of actin during steady-state ATP hydrolysis (Ostap and Thomas, 1991). Therefore, we concluded that the observed microsecond rotational motions are due to the rotation of S1 relative to actin. An alternative explanation for the observed microsecond rotational motions of MSL-S1 bound to actin in the presence of ATP is that the spin label may become mobilized relative to the myosin head. While nucleotide binding to myosin has been known to mobilize certain spin labels (Barnett and Thomas, 1987; Ostap et al., 1993) and fluorescent probes (Thomas, 1987; Tanner et al., 1992), MSL-S1 fixed on glass beads has been shown to remain rotationally rigid on the microsecond time scale in the presence of ATP, indicating that mobilization of MSL relative to the myosin head upon nucleotide binding is not occurring (Thomas et al., 1980). To determine even more rigorously whether the whole myosin head is rotating and contributing to these motions, or just the region around SH1 and SH2, it will be necessary to perform complementary experiments with the spin-label at other sites on S1.

While it is possible that the rapid attachment and detachment cycle of myosin heads with actin in the presence of ATPγS affects the ST-EPR spectrum, stiffness measurements on single muscle fibers in the presence of 5 mM MgATPγS indicate that the crossbridge detachment rate is on the order of 10^3 s^{-1} (Dantzig and Goldman, 1988). Thus, the observed rotational motions of the A.M.ATPγS ternary complex are too fast to be accounted for by the rapid association/dissociation of myosin heads with actin. It is unlikely that spin-labeling increases the detachment rate of crossbridges from actin in the presence of ATPγS, since there was no significant difference in the fraction of actin-attached myosin heads at physiological ionic strength in the presence of ATPγS in myofibrils or in solutions of acto-S1 (data not shown).

We argued above that 5 mM ATPγS is sufficient to saturate the myofibrillar myosin active sites even in the presence of Ca^{2+}. Even if this were not true, and some nucleotide-free rigor crossbridges remained (as suggested by Kraft et al., 1992), this could only produce a bound spectrum (Fig. 4, *right column*) corresponding to decreased rotational mobility. Thus the A.M.ATPγS complex could be more mobile than we have concluded, but it could not be less mobile. This

further validates our conclusion that a substantial portion of actin attached myosin heads in a ternary complex with MgATPγS are rotationally mobile.

If all actin-attached myosin heads are not equally protected from proteolysis at T_2, a lower limit for the actual fraction of actin-attached myosin heads would be obtained in the tryptic digestion binding experiments. However, this would not seriously affect the interpretation of our data, even in the unlikely event that all of the myosin heads in the presence of 16 mM MgAMPPNP or 5 mM MgATPγS are, in reality, attached to actin. In this worst-case scenario, the myosin heads complexed with actin and MgAMPPNP would still have only restricted microsecond rotational motions, and those complexed with actin and MgATPγS would have almost as much rotational motion as detached myosin heads. Thus, there would be no qualitative change in our conclusion that myosin heads in weakly bound states have at least an order of magnitude more rotational motion on the microsecond time scale (as judged by τ_r) than those in strongly bound states.

It has been suggested by Geeves et al. (1984) that all biochemical states of the actomyosin ATPase cycle exist in weak- and strong-binding conformations and that the equilibrium between these conformations is shifted toward weak binding for states early in the ATPase cycle and toward strong binding in the late states of the ATPase cycle. Thus, there may be two populations of actin-attached myosin heads complexed with MgAMPPNP or ATPγS: a strongly bound and immobile fraction and a weakly bound and rotationally mobile fraction. However, our results show that the fraction of mobile heads in A.M.AMPPNP is negligible, and the fraction of immobile heads in A.M.ATPγS is no more than 64%. Conventional EPR experiments on spin-labeled muscle fibers in the presence of MgATPγS will be necessary to examine the orientational distribution of the myosin heads to resolve this point. Previous EPR experiments (Baker and Cooke, 1988) have shown a small oriented population of myosin heads in the presence of 1 mM MgATPγS, but the nucleotide concentration was sufficiently low that the myosin heads were probably not saturated with nucleotide.

Spin-labeling affects the fraction of actin-attached myosin heads in active myofibrils (Berger and Thomas, 1993) but not in the presence of ATPγS. The effect of spin-labeling is to shift the population of crossbridges from strongly bound states of the actomyosin ATPase cycle to weakly bound ones in active myofibrils, rather than to affect the actomyosin affinity directly (Matta and Thomas, 1992; Ostap et al., 1993). It may also be that the strongly bound intermediates of the myosin ATPase cycle are not statically attached to actin but are rotationally mobile on the millisecond time scale and are too slow to be detected by ST-EPR. Time-resolved spectroscopic experiments on the millisecond time scale may be useful in addressing this issue.

CONCLUSIONS

We have demonstrated that, within the myofibrillar lattice, independent of ionic strength, myosin heads attached to actin in the presence of ATPγS are rotationally mobile on the microsecond time scale, while those in the presence of AMPPNP are not. These results suggest that at least some of the early, weakly bound intermediates of the myosin ATPase cycle are dynamically attached to actin, while the strongly bound, late intermediate states are not. Therefore, we propose that force generation involves a crossbridge transition from dynamic rotational disorder to rigid, rigor-like order. The microsecond rotational motions observed for actin-attached myosin heads in the presence of ATPγS are quite similar to those in active spin-labeled myofibrils (Berger and Thomas, 1993) and isometric muscle fibers (Barnett and Thomas, 1989), but it is not yet possible to differentiate the rotational motion of pre-force and force-generating states during the active actomyosin ATPase cycle. Thus, although it is now clear that actin-attached myosin heads in the actomyosin ATPase cycle are rotationally mobile on the microsecond time scale, even within the myofibrillar lattice at physiological ionic strength, the challenge remains to correlate these motions directly with the processes involved in shortening and force generation in muscle.

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