Rotational Dynamics of Actin-Bound Myosin Heads in Active Myofibrils†

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ABSTRACT: We have used saturation-transfer electron paramagnetic resonance (ST-EPR) to measure the submillisecond rotational motions of actin-bound myosin heads in active myofibrils. The cross-bridges were spin-labeled with a maleimide nitroxide derivative (MSL) that has previously been shown to undergo microsecond rotational motions on actin-bound myosin heads in solution during steady-state ATPase activity at low ionic strength [Berger, C. L., Svensson, E. C., & Thomas, D. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8573]. To determine whether this is also true for cross-bridges in the myofibrillar lattice under physiological buffer conditions, we have performed ST-EPR experiments during the brief steady state following photolysis of caged ATP in a suspension of spin-labeled myofibrils. The myofibrils were partially cross-linked with EDC [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide] to prevent their shortening upon activation. The fraction of actin-attached myosin heads was determined biochemically at physiological ionic strength in the active myofibrils, using the proteolytic rates acto–myosin binding assay [Duong, A. M., & Reisler, E. (1989) *Biochemistry 28*, 3502]. These data were then used to correct the ST-EPR spectra of active myofibrils for the presence of unattached myosin heads, which were assumed to undergo the same motions as in relaxation. At physiological ionic strength (μ = 165 mM), actin-bound myosin heads were found to have considerable microsecond rotational motion (τ_r = 3.5 ± 1.1 μs) in the active myofibrils. Similar results (τ_r = 3.2 ± 0.8 μs) were obtained with active myofibrils at low ionic strength (μ = 45 mM), confirming the work done in solution. Thus, under physiological conditions and even within the constraints of the myofibrillar lattice, actively cycling actin-attached myosin heads are rotationally mobile on the microsecond time scale. Since partially EDC-fixed myofibrils are an excellent analog of isometrically contracting muscle fibers in solution, it is likely that these microsecond rotational motions are directly related to the molecular mechanism of muscle contraction in vivo.

Muscle contraction involves the cyclic interaction between actin and myosin, whereby the chemical free energy of adenosine 5′-triphosphate (ATP)[1] hydrolysis is converted into mechanical work (Lymn & Taylor, 1971; Eisenberg & Hill, 1985). In particular, force generation and myofilament sliding are thought to occur via conformational changes in the myosin head (subfragment 1, S1), while bound to actin during the acto–myosin ATPase cycle (Huxley, 1969; Huxley & Simmons, 1971; Huxley & Kress, 1985). Therefore, direct measurements of S1's structural dynamics during the contractile process are imperative for understanding the molecular mechanism of muscle contraction. Spectroscopic techniques such as electron paramagnetic resonance (EPR) and time-resolved phosphorescence anisotropy (TPA) provide excellent means by which to monitor protein motions at specific sites in macromolecular complexes such as acto–myosin [reviewed by Thomas (1987)].

Previously, conventional EPR, used to measure cross-bridge orientation, and saturation-transfer EPR (ST-EPR), used to measure cross-bridge microsecond rotational motion, of isometrically contracting skinned skeletal muscle fibers have demonstrated that most (80–90%) of the myosin heads are highly disordered (Cooke et al., 1982; Fajer et al., 1990) and mobile on the microsecond time scale (Barnett & Thomas, 1989). TPA experiments have also indicated that cross-bridges in isometrically contracting skinned muscle fibers are dynamically disordered on the microsecond time scale, in a state distinct from either rigor or relaxation (Stein et al., 1990). Since stiffness measurements suggest that greater than 50% of the myosin molecules are attached to actin under these conditions, it appears likely that even the myosin heads attached to actin are undergoing microsecond rotational motions (Barnett & Thomas, 1989; Fajer et al., 1990). However, active muscle fiber stiffness is not necessarily a linear function of the fraction of actin-bound myosin heads (Fajer et al., 1988; Pate & Cooke, 1988). Stiffness measurements cannot differentiate between single or double myosin head attachment to actin, and the assumption that the stiffness in an active cross-bridge is equal to that of a rigor cross-bridge may not be valid. Thus, it is difficult to resolve the rotational motions arising solely from the actin-attached myosin heads from those of the detached myosin heads in the muscle fiber.

In solutions of purified actin and S1, the fraction of actin-bound myosin heads can be determined unambiguously from centrifuge binding assays (Chalovich & Eisenberg, 1982). Previous ST-EPR experiments, in conjunction with biochemical centrifuge binding assays, on solutions of acto–S1 have directly demonstrated the rotational mobility of S1 while bound

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; caged ATP, P^3-1-(2-nitrophenyl)ethyl ester of ATP; S1, myosin subfragment 1; HMM, heavy meromyosin; MSL, 4-maleimido-2,2,6,6-tetramethyl-1-piperidyloxy; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer electron paramagnetic resonance; SEM, standard error of the mean; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $MgCl_2$, magnesium chloride; KPr, potassium propionate; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; βME, β-mercaptoethanol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Vi(Na_3VO_4), sodium vanadate; $CaCl_2$, calcium chloride.

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to actin during the ATPase cycle (Berger et al., 1989). However, with solutions of acto–S1, the myosin head is not constrained within the myofibrillar lattice by its attachment to the thick filament backbone as it is in the muscle fiber. Furthermore, the affinity of S1 for actin in the presence of MgATP at physiological ionic strength is quite weak, and solution studies are usually done at very low, nonphysiological ionic strengths in order to increase the acto–myosin affinity in the presence of MgATP. ST-EPR experiments of EDC-cross-linked acto–S1 have shown actin-attached S1 to be rotationally mobile on the microsecond time scale in the presence of MgATP at physiological ionic strength (Svensson & Thomas, 1986), but it is not clear that cross-linked myosin heads represent a physiological attachment of actin and S1. Therefore, in order to bridge the gap between solution studies (in which the acto–myosin interaction can be well characterized) and the more physiologically relevant skinned muscle fiber preparations, in the present study we have used ST-EPR to monitor the microsecond rotational motions of myosin heads attached to actin in active myofibrils.

Partial cross-linking of myofibrils with EDC prevents them from shortening in the presence of ATP and calcium, resulting in an excellent model system of isometrically contracting muscle fibers in solution (Glyn & Sleep, 1985). Both the steady-state ATPase rate (Glyn & Sleep, 1985) and the rate of oxygen exchange via labeled phosphate (Bowater & Sleep, 1985) in EDC-cross-linked myofibrils are similar to values obtained with isometrically contracting skinned muscle fibers. In myofibrils, but not in muscle fibers, the fraction of actin-attached myosin heads can be determined directly from a tryptic digestion assay, even during isometric contraction at physiological ionic strength (Duong & Reisler, 1989). The myosin heavy chain contains a tryptic cleavage site at the 50K/20K junction that is protected when myosin is bound to actin, but not when it is detached (Lovell & Harrington, 1981). Thus, the rate of tryptic digestion of the myosin heavy chain in active myofibrils can be compared to that of myofibrils in rigor (in which all myosin heads are attached to actin), and in relaxation (in which all myosin heads are detached from actin), to determine the fraction of actin-bound myosin heads (Duong & Reisler, 1989). In EPR and ST-EPR measurements during active cycling in the presence of MgATP, only a brief period of time (less than 1 min) is available for spectroscopic data acquisition (Berger et al., 1989; Fajer et al., 1990). Therefore, as in those previous studies, active contraction of the myofibrils was initiated by the photolysis of caged ATP, and the spectral intensity was monitored at a single field position that is maximally sensitive to microsecond rotational motions. Thus, using ST-EPR in conjunction with the tryptic digestion binding assay, we were able to detect directly the microsecond rotational motion of spin-labeled myosin heads bound to actin in active myofibrils under physiological conditions.

MATERIALS AND METHODS

Preparations and Solutions. MSL (4-maleimido-2,2,6,6-tetramethyl-1-piperinyloxy) was obtained from Aldrich. DTT (dithiothreitol) was obtained from Boehringer-Mannheim. ATP (adenosine 5′-triphosphate), ADP (adenosine 5′-diphosphate), EDC [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide], βME (β-mercaptoethanol), trypsin, soybean trypsin inhibitor, and DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] were obtained from Sigma. Sodium vanadate (Na_3VO_4) was obtained from Fisher Scientific and prepared as described previously (Barnett & Thomas, 1987). Caged ATP was acquired from Calbiochem. All other chemicals were of reagent grade and of the highest quality available. Experimental solutions (pH 7.0, 25 °C) contained 25 mM imidazole, 7 mM $MgCl_2$, 1 mM EGTA, 2 mM $CaCl_2$, and 5 mM caged ATP. Rigor solutions contained no nucleotide, and relaxed solutions contained no $CaCl_2$, and either 3 mM MgADP·V_i + 0.1 mM MgATP or 5 mM caged ATP. Vanadate solutions were prepared as previously described (Goodno, 1980; Barnett & Thomas, 1987). The desired ionic strengths were acquired with the addition of the appropriate amounts of potassium propionate (KPr).

Myofibrils were prepared from MSL-labeled and unlabeled glycerinated rabbit skeletal muscle (psoas) fibers as described previously (Ludescher & Thomas, 1988). Myofibrils were stored in a low ionic strength rigor buffer (25 mM imidazole, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.0) and were always used within 2 days of preparation. The glycerinated muscle fibers were spin-labeled essentially as described previously (Fajer et al., 1988), except that fibers were not treated with $K_3Fe(CN)_6$, but were pretreated with the reversible sulfhydryl reagent DTNB in order to block cysteines other than Cys-707 (SH_1) from reacting with MSL during the labeling procedure. Fibers were incubated in rigor buffer (130 mM KPr, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, and 20 mM MOPS, pH 7.0) with 60 μM DTNB prior to spin-labeling with MSL. After 60 min, the excess DTNB was removed by three washes with rigor buffer. After the spin-labeling reaction (Fajer et al., 1988) was complete, all DTNB was removed from the muscle fibers by incubation with 10 mM DTT in rigor buffer, followed by three washes of rigor buffer.

The fraction of myosin heads labeled at SH_1 (Cys-707) and/or SH_2 (Cys-697) in the MSL-myofibrils was determined to be $f_{SH} = 0.96 \pm 0.03$ from fractional inhibition of the high-salt K/EDTA-ATPase activity of myofibrils (Ludescher et al., 1988), and the total spin-labeling extent was determined to be $f_{SL} = 1.20 \pm 0.06$ spin-labels bound per myosin head, from the double integration of the conventional EPR spectrum (Thomas et al., 1980). Thus, the specificity of labeling (fraction of bound labels reacting with myosin SH_1 or SH_2) was $f_{SH}/f_{SL} = 0.80 \pm 0.07$. The concentration of protein in myofibril suspensions was determined by the biuret assay (Gornall et al., 1949), using bovine serum albumin as a standard. The concentration of myosin heads was calculated by assuming that 50% of the protein in myofibrils is myosin, which has a molecular weight of 480 000 (Ludescher et al., 1988). Myofibrils (MSL-labeled and unlabeled) were cross-linked in rigor with EDC as described previously (Duong & Reisler, 1989). Briefly, myofibrils were adjusted to 1 mg/mL in 0.1 M NaCl/0.1 M MES, pH 6.5 (4 °C), and EDC was added to a final concentration of 10 mM. The myofibrils were constantly stirred on ice for 30 min during the cross-linking reaction. The reaction was stopped with the addition of 0.3 M βME, and the myofibrils were washed several times with low ionic strength myofibril storage buffer. The steady-state ATPase activity of myofibrils was assayed as described previously (Ludescher & Thomas, 1988). The myofibrillar suspension (0.2–0.5 mg/mL) was continuously stirred in a 1-mL incubation vial containing the appropriate buffer (identical to the corresponding buffer used for EPR and proteolytic digestion), in a thermostatically controlled water bath at 25 °C. The reaction was initiated by the addition of 5 mM ATP, and small (usually 50 μL) aliquots were removed at 1-min intervals and assayed for inorganic phosphate by the method of Lanzetta et al. (1979). The steady-state rate of phosphate production [reported as international units (IU),

micromoles per milligram of protein per minute] was determined by a linear regression analysis of the data from the linear phase of the reaction during the first 2–4 min of the incubation.

Tryptic Digestion Experiments. In order to determine the fraction of actin-attached myosin heads within the active myofibrils, tryptic digestion experiments were done by the method of Duong and Reisler (1989). The myosin heavy chain has three tryptic cleavage sites: one between the 25K and 50K domains of S1 (T_1), another between the 50K and 20K domains of S1 (T_2), and a third 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 tryptic digestion at T_2 is very dependent on the state of the myosin head. When detached, 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 225K heavy chain, the 200K product cleaved at T_1, and the 150K product cleaved at T_2. In a mixture of attached and detached cross-bridges, the fraction of actin-bound myosin heads (f_B) could, in principle, be determined from the relative proportions of these three digestion products. However, since the cross-bridges in active myofibrils are usually in a rapid equilibrium between the attached and detached states, all of the myosin heads will eventually be cleaved at T_2. Therefore, it is necessary to measure the initial rates of tryptic digestion at T_2, which will be directly related to the fraction of actin-bound (f_B) and free (f_F) myosin heads in the myofibril:

$$f_B = \frac{k_{2D} - k_{2M}}{k_{2D} - k_{2A}} \quad (1)$$

$$f_F = \frac{k_{2M} - k_{2A}}{k_{2D} - k_{2A}} \quad (2)$$

where k_{2A} and k_{2D} are the tryptic rates of digestion at T_2 of actin-attached and detached myosin heads, respectively, and k_{2M} is the rate of tryptic digestion at T_2 for an equilibrium mixture of actin-attached and detached myosin heads. This expression assumes that the rates of association and dissociation between actin and myosin are much greater than k_{2A} and k_{2D}, so that an equilibrium population of the myosin heads is sampled.

Myofibrils were adjusted to 0.5 mg/mL in a total volume of 1.0 mL and digested with 0.01 mg/mL trypsin for 5 min in the same buffer used for EPR spectroscopy. At this concentration of trypsin, the rate of tryptic digestion at T_2 is several orders of magnitude slower than the acto–myosin rates of association and dissociation (Duong & Reisler, 1989). 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 (50 μL) 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 fraction of myosin heads bound to actin (f_B) in the myofibrils was then determined from eq 2; k_{2A} was determined from the tryptic digestion of rigor myofibrils, k_{2D} was determined from the tryptic digestion of EDC-cross-linked myofibrils relaxed at physiological ionic strength (μ = 165 mM) with 5 mM MgADP·V_i and 0.1 mM MgATP in the absence of Ca^{2+}, and k_{2M} was determined from the tryptic digestion of active EDC-cross-linked myofibrils in the presence of 2 mM Ca^{2+}. Normal relaxing conditions (5 mM MgATP, no Ca^{2+}) were not sufficient to measure k_{2D} since the thin filaments become activated due to the tryptic degradation of troponin C (Duong & Reisler, 1989).

The intensities of the 225K, 200K, and 150K bands of the myosin heavy chain, as well as the 42K actin band, were determined by scanning densitometry for each sample after it had been run on SDS/PAGE. The intensities of the myosin heavy-chain 225K and 220K bands were monitored as a function of time, and the resulting decays were 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 in order to correct for differences in dye absorption, and all myosin heavy-chain bands were normalized to the intensity of the actin band to account for any variations in gel loading between the different samples.

ST-EPR Experiments. EPR and ST-EPR spectra were obtained using a Bruker ESP 300 spectrometer equipped with a TE_{102} cavity with an optical port. 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 base lines were 100 G wide, except for the transient ST-EPR experiments, which measured the spectral field intensity at a single field position in the center of the spectrum (where the sensitivity to rotational motion is maximal) as a function of time. To ensure that the transient EPR signal was always obtained at an equivalent spectral position, the ratio of the applied magnetic field to the microwave frequency was locked at a constant value, using the field/frequency lock of the spectrometer. The accuracy and reproducibility of this lock were verified by reference to the base-line-crossing points of 0.9 mM peroxylamine disulfonate (PADS; Alfa Products) in a deoxygenated (nitrogen-saturated) solution containing 50 mM K_2CO_3. The temperature was maintained at 25 °C by flowing precooled N_2 over the sample, which was regulated by a variable-temperature controller (Bruker). Myofibril samples were contained in a fused silica tissue cell with TPX coverplate (Wilmad Glass Co.). Caged ATP was photolyzed during EPR experiments with a 50-ms burst (5 pulses at 100 Hz) from a XeF excimer laser (Lambda Physik) at 351 nm, introduced directly into the optical port of the EPR cavity. Light energy incident on the sample, measured with a light meter connected to a fiber optic detector, was approximately 150 mJ/cm^2 for a single 100-Hz pulse; 1–2 mM ATP was photoreleased per 5-pulse burst, as determined by assaying the amount of inorganic phosphate produced by the acto–myosin hydrolysis reaction, using the method of Lanzetta et al. (1979). Previous control experiments ruled out nonspecific effects of UV irradiation on our data (Berger et al., 1989; Fajer et al., 1990). Myofibril ST-EPR samples typically contained 20–30 mg of total protein, corresponding to approximately 40–60 μM MSL-labeled myosin heads.

Digitized EPR spectra (1024 points per scan) were acquired with the ESP 300 spectrometer's built-in Bruker OS-9-compatible ESP 1620 spectral acquisition software, and then transferred to an IBM-compatible microcomputer. All ST-

Table I: Myofibril Tryptic Digestion Binding and ST-EPR Experimental Results[a]

	IS (mM)	$k_{td} \times 10^{-3}$ (s^{-1})	f_B	ST-EPR int.	τ_r (μs)
rigor					
EDC-MSL-myofibrils	165	0.00 ± 0.03	≡1	≡1	>100
EDC-MSL-myofibrils	45	0.00 ± 0.08	≡1	≡1	>100
relaxed					
MSL-myofibrils	165		≡0	0.47 ± 0.01	1.8 ± 0.1
EDC-MSL-myofibrils	165	4.85 ± 0.11	≡0	0.50 ± 0.01	2.1 ± 0.1
active					
EDC-MSL-myofibrils	165	3.68 ± 0.06	0.24 ± 0.02	0.54 ± 0.02	2.5 ± 0.2
EDC-MSL-myofibrils	45	3.07 ± 0.14	0.37 ± 0.04	0.57 ± 0.02	2.8 ± 0.4
actin-bound population					
EDC-MSL-myofibrils	165		≡1	0.64 ± 0.09	3.2 ± 0.8
EDC-MSL-myofibrils	45		≡1	0.66 ± 0.13	3.5 ± 1.1

[a] IS is ionic strength. k_{td} is the rate of tryptic digestion, which is used to determine f_B, the fraction of myosin heads bound to actin (eq 1). ST-EPR int. is the normalized spectral intensity detected in caged ATP experiments. τ_r^{eff}, the effective rotational correlation time, was determined from ST-EPR int. The value of ST-EPR int. for the actin-bound population was determined using eq 4.

EPR spectra were analyzed using a program developed by Robert L. H. Bennett. Four to sixteen 200-s scans were typically averaged together for the steady-state ST-EPR spectra. Only one scan was acquired for each transient ST-EPR spectrum. 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 & Thomas, 1986). In the transient ST-EPR experiments, values of τ_r were determined from the spectral intensity of the single field position monitored, normalized to the rigor value. Calibration curves, derived from spin-labeled hemoglobin samples with known rotational correlation times, were used to determine τ_r in both the steady-state (Thomas et al., 1976; Squier & Thomas, 1986) and transient ST-EPR experiments (Berger et al., 1989).

The ST-EPR spectral intensity of the spin-labeled myofibrils (S_{tot}) during steady-state cycling of the acto–myosin ATPase will be a composite of the ST-EPR spectral intensity of actin-bound myosin heads (S_{bnd}) and the detached myosin heads (S_{det}) weighted by their respective mole fractions (f_{bnd}, f_{det}):

$$S_{tot} = f_{bnd}S_{bnd} + f_{det}S_{det} \quad (3)$$

Thus, the ST-EPR spectral intensity of the actin-bound myosin heads (S_{bnd}) can be explicitly determined by rearrangement of eq 3:

$$S_{bnd} = \frac{S_{tot} - f_{det}S_{det}}{f_{bnd}} \quad (4)$$

where f_{det} and f_{bnd} are the fractions of free (f_F) and actin-bound (f_B) myosin heads determined from the tryptic digestion experiments (eq 1 and 2), and S_{det} is the ST-EPR spectral intensity of relaxed EDC-MSL-myofibrils. It was assumed that the contribution of the cross-linked myosin heads in both the active and the relaxed EDC-MSL-myofibrils was the same. ST-EPR spectra were acquired for both EDC-cross-linked and un-cross-linked relaxed MSL-myofibrils, and the difference in spectral intensity between these two samples was then subtracted from the spectral intensity of the active EDC-MSL-myofibril to correct for the contribution of the cross-linked myosin heads. Thus, the spectral intensities of the un-cross-linked relaxed and the corrected EDC-cross-linked active MSL-myofibrils were used in eq 4 to determine the spectral intensity of the un-cross-linked actin-bound myosin heads in the active EDC-MSL-myofibrils.

RESULTS

Myofibrillar ATPase Assays. The steady-state ATPase activity of calcium-activated MSL-myofibrils (2.9 ± 0.4 s^{-1}) at physiological ionic strength (μ = 165 mM) is 80% of the activity of unlabeled myofibrils (3.6 ± 0.3 s^{-1}). More than 90% of the myosin heads are spin-labeled in this preparation. Therefore, while it is known that modification of SH_1 (Cys-707) on the myosin heavy chain alters the myosin kinetic cycle, the spin-labeled heads are still capable of undergoing a complete ATPase cycle (Svensson & Thomas, 1986; Matta & Thomas, 1992). EDC cross-linking increased the ATPase activity of MSL myofibrils to 3.7 ± 0.3 s^{-1}. This modest increase is consistent with previous studies, which have shown that only a small fraction (<25%) of the myosin heads are cross-linked to actin in this preparation (Duong & Reisler, 1989; Glyn & Sleep, 1985). Nevertheless, this level of cross-linking was sufficient to prevent active shortening of the MSL-myofibrils, as observed by phase-contrast microscopy. Spin-labeling increased the relaxed myofibrillar ATPase approximately 20-fold (from 0.09 ± 0.02 to 1.9 ± 0.4 s^{-1}), in agreement with previous studies that have demonstrated desensitization of the acto–myosin ATPase to Ca^{2+} with modification of SH_1 (Titus et al., 1987; Matta & Thomas, 1992). The ATPase activity of active EDC-MSL-myofibrils was low enough that ATP depletion did not occur during the tryptic digestion experiments, due to the relatively short assay time (5 min) and low myofibril concentration (0.5 mg/mL) used.

Myofibril Tryptic Digestion Binding Experiments. EDC-MSL-myofibrils were digested with trypsin in order to determine the fraction of myosin heads bound to actin during the steady-state hydrolysis of MgATP (Duong & Reisler, 1989). The rate of tryptic digestion at the 50K/20K junction of the myosin head (k_{2M}) under active conditions was compared with reference rates obtained from rigor (k_{2A}) and relaxed (k_{2D}) EDC-MSL-myofibrils (eq 1 and 2). These results are summarized in Table I and Figure 1. Relaxed EDC-MSL-myofibrils were digested at physiological ionic strength (μ = 165 mM) in the presence of 3 mM MgADP·V_i and 0.1 mM MgATP, conditions in which all of the myosin heads are detached from actin, even if the thin filament becomes partially activated due to tryptic digestion of troponin C (Duong & Reisler, 1989). The rate of tryptic digestion of the relaxed EDC-MSL-myofibrils was k_{2D} = (4.85 ± 0.11) × 10^{-3} s^{-1}. In rigor EDC-MSL-myofibrils, the rate of tryptic digestion was not detectable at low (μ = 45 mM) or physiological (μ = 165 mM) ionic strength, indicating that all the myosin heads were bound to actin (k_{2A} = 0). Under active conditions (15 mM

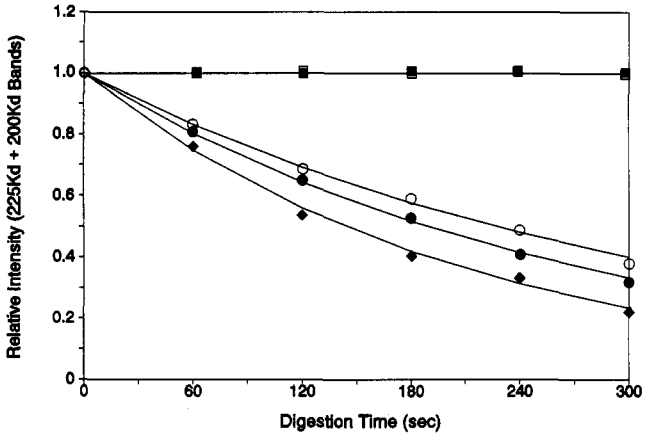

FIGURE 1: Rate of tryptic digestion of EDC-MSL-myofibrils. The intensity of the myosin heavy chain (225-kDa + 200-kDa bands) is plotted as a function of tryptic digestion time. Values have been normalized to the initial intact heavy-chain intensity. Rates of tryptic digestion were determined from a single-exponential 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. (♦) EDC-MSL-myofibrils relaxed at physiological ionic strength (μ = 165 mM) with 3 mM MgADP·V_i + 0.1 mM MgATP. (■) Rigor EDC-MSL-myofibrils at physiological ionic strength (μ = 165 mM). (□) Rigor EDC-MSL-myofibrils at low ionic strength (μ = 45 mM). (●) Active EDC-MSL-myofibrils at physiological ionic strength. (○) Active EDC-MSL-myofibrils at low ionic strength (μ = 45 mM).

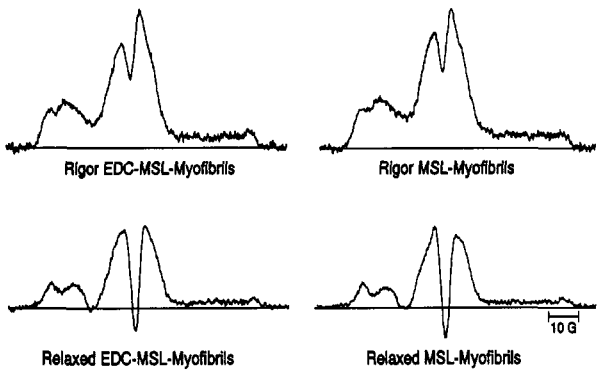

FIGURE 2: ST-EPR spectra of MSL-myofibrils (right) and EDC-MSL-myofibrils (left) at physiological ionic strength (μ = 165 mM) relaxed with 3 mM MgADP·V_i + 0.1 mM MgATP (bottom) or in the absence of nucleotide (rigor, top). Samples contained 20–30 mg/mL MSL-myofibrils or EDC-MSL-myofibrils. Spectral base lines are 100 G wide.

MgATP, 2 mM Ca^{2+}) at physiological ionic strength, the EDC-MSL-myofibrils were digested at a rate of k_{2M} = (3.68 ± 0.06) × 10^{-3} s^{-1}. Substituting these rate constants into eq 1 yields a value of f_B = 0.24 ± 0.02 for the fraction of myosin heads bound to actin under active conditions. At low ionic strength, the rate of tryptic digestion in active EDC-MSL-myofibrils was (3.07 ± 0.14) × 10^{-3} s^{-1}, corresponding to 0.37 ± 0.04 of the myosin heads being bound to actin. The rates of tryptic digestion (k_{2D}, k_{2A}, and k_{2M}) were also measured for *unlabeled* EDC-myofibrils at physiological ionic strength. These results indicated that the only rate significantly affected by the label was that of the active state [k_{2M} = (3.25 ± 0.13) × 10^{-3} s^{-1}], indicating a larger fraction (0.33 ± 0.03) of actin-bound myosin heads in the absence of the spin-label.

ST-EPR Spectra in Rigor and Relaxation. The ST-EPR spectra of myofibrils in rigor (Figure 2, bottom) have the line shape and high intensity characteristic of very strongly immobilized spin-labels, indicating that the myosin heads in these preparations are rigidly bound to actin. Due to the rather long time period (20–30 min) required to acquire a full

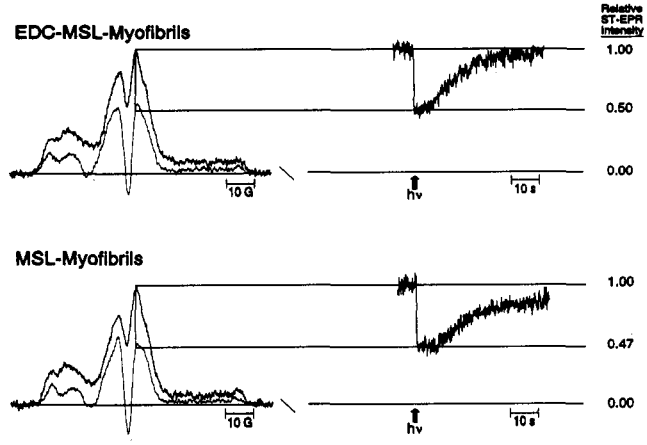

FIGURE 3: Effect of the photolysis of caged ATP on the ST-EPR intensity of MSL-myofibrils (bottom) and EDC-MSL-myofibrils (top) at physiological ionic strength (μ = 165 mM) under relaxing conditions (no $CaCl_2$ added). Left: ST-EPR spectra of rigor MSL-myofibrils (bottom) and EDC-MSL-myofibrils (top) in the presence of 5 mM caged ATP before photolysis, and relaxed MSL-myofibrils and EDC-MSL-myofibrils, showing the spectral position at which the magnetic field was fixed. Right: ST-EPR spectral intensity at fixed magnetic field position as a function of time before and after photolysis of caged ATP (arrow). Samples contained 20–30 mg/mL MSL-myofibrils or EDC-MSL-myofibrils.

ST-EPR spectrum, and the high concentration of myofibrils (20–30 mg/mL) required for adequate signal intensity, EDC-MSL-myofibrils and MSL-myofibrils were relaxed either at physiological ionic strength (μ = 165 mM) in the presence of 3 mM MgADP·V_i and 0.1 mM MgATP (to obtain the entire spectrum, as in Figure 2, bottom) or following flash photolysis of caged ATP (to obtain the intensity at a single spectral position in the brief steady-state period). The ST-EPR spectra of relaxed myofibrils (Figure 2, bottom) have the line shape and low intensity that indicates considerable microsecond rotational motion. EDC cross-linking had no effect on the spectrum in rigor, but it resulted in slightly decreased rotational mobility in relaxation (Figure 2, left).

ST-EPR Transients in Relaxation. Prior to photolysis, the ST-EPR spectra in rigor were unaffected by 5 mM caged ATP (Figure 3). In the transient EPR experiments, a single spectral field position, which has been shown to be highly sensitive to changes in microsecond rotational motion and has maximal intensity for the rigor acto–myosin complex (Berger et al., 1989), was monitored. The ST-EPR intensities of relaxed myofibrils following the photolysis of caged ATP (Figure 3) were identical to those at the same field position in the ST-EPR spectra in relaxation (Figure 2). Upon relaxation, 1–2 mM caged ATP was photolyzed, and the ST-EPR intensity (normalized to rigor) rapidly decreased to 0.47 ± 0.01 for un-cross-linked myofibrils, indicating increased microsecond rotational motions of the myosin heads as they detached from actin (Figure 3, Table I). For cross-linked myofibrils, the spectral intensity only decreased to 0.50 ± 0.01 (Figure 3, Table I), indicating slightly less rotational motion than for un-cross-linked myofibrils, presumably due to the decreased mobility of the small fraction of heads that were cross-linked to actin. Since the rotational motion of myosin heads cross-linked by EDC, either to the thick filament backbone or to actin, is independent of [Ca^{2+}], this difference (0.03 of the rigor intensity) was used to correct ST-EPR spectra of active EDC-MSL-myofibrils for the contribution of the cross-linked myosin heads.

ST-EPR Transients in Activation. In the presence of 2 mM Ca^{2+}, the ST-EPR spectra of EDC-MSL-myofibrils

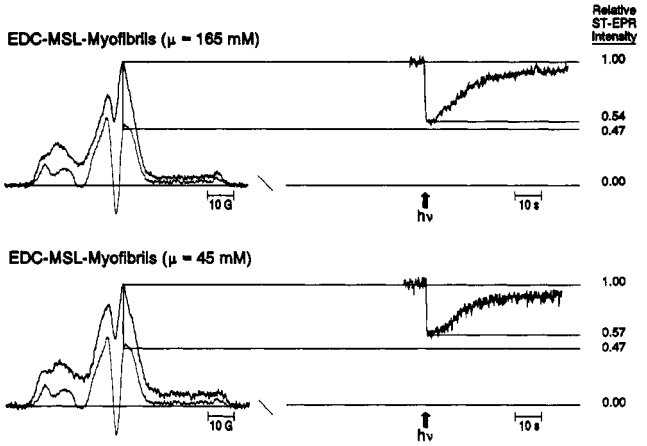

FIGURE 4: Effect of the photolysis of caged ATP on the ST-EPR intensity of EDC-MSL-myofibrils at physiological (μ = 165 mM; top) and low (μ = 45 mM; bottom) ionic strengths under active conditions (2 mM $CaCl_2$ added). Left: ST-EPR spectra of rigor EDC-MSL-myofibrils in the presence of 5 mM caged ATP before photolysis, and relaxed EDC-MSL-myofibrils, showing the spectral position at which the magnetic field was fixed. Right: ST-EPR spectral intensity at fixed magnetic field position as a function of time before and after photolysis of caged ATP (arrow). Samples contained 20–30 mg/mL EDC-MSL-myofibrils.

indicated that the myosin heads were rigidly bound to actin prior to photolysis in the presence of 5 mM caged ATP, regardless of ionic strength (Figure 4). Upon photolysis, 1–2 mM ATP was released into the sample, producing activation, and a decrease in the spectral intensity to a brief steady-state value was observed at both physiological (165 mM) and low (45 mM) ionic strengths (Figure 4). The spectral intensity dropped almost as much under active conditions (Figure 4) as under relaxing conditions (Figure 3), but the small differences in spectral intensity among the three sets of conditions were significant ($p < 0.01$). This level was the true steady state at saturating [ATP], since a more intense light pulse (producing higher [ATP] and a longer steady-state phase) resulted in the same minimum ST-EPR intensity. The spectral intensity (normalized to rigor) of active EDC-MSL-myofibrils dropped to 0.54 ± 0.02 at physiological ionic strength, and to 0.57 ± 0.02 at low ionic strength. After depletion of ATP, the ST-EPR spectral intensity consistently returned to the original rigor ST-EPR intensity level, indicating a normal acto–myosin interaction. Although active cross-bridges are only slightly less rotationally mobile than relaxed cross-bridges, binding measurements indicate a large difference in the fraction of actin-attached myosin heads. Effective rotational correlation times (τ_r) were determined using a calibration curve of MSL-hemoglobin samples with known values of τ_r at the same single ST-EPR field position (Berger et al., 1989). The value of τ_r determined from this intensity parameter was 1.8 ± 0.1 μs for relaxed MSL-myofibrils, and 2.1 ± 0.1 μs for relaxed EDC-MSL-myofibrils. The value of τ_r for active EDC-MSL-myofibrils was 2.5 ± 0.2 μs at physiological ionic strength, and 2.8 ± 0.4 μs at low ionic strength.

Rotational Motion of Actin-Bound Heads. The ST-EPR spectral intensity of the MSL-myofibrils during contraction is a linear combination of the actin-attached and detached myosin heads, weighted by their mole fractions (eq 3). Therefore, we used eq 4 to calculate the ST-EPR spectral intensity for the actin-attached component of the myosin heads in active EDC-MSL-myofibrils and MSL-myofibrils, using the values of actin-attached (f_B) and detached (f_F) myosin heads obtained from the tryptic digestion binding experiments (eq 1 and 2), and assuming that the rotational motion of detached myosin heads in active myofibrils is equivalent to the rotational motion of myosin heads during relaxation. The ST-EPR spectral intensity of the active, actin-bound myosin heads in EDC-MSL-myofibrils was determined to be 0.64 ± 0.09 at physiological ionic strength and 0.66 ± 0.13 at low ionic strength, corresponding to effective rotational correlation times of 3.2 ± 0.8 and 3.5 ± 1.1 μs, respectively. The rotational motion of actin-attached myosin heads in active myofibrils appears only slightly more restricted than that in solution with acto–S1 at low ionic strength (Berger et al., 1989), and is statistically different from that of relaxed ($p < 0.01$) and rigor ($p < 0.001$) myosin heads at either low or physiological ionic strength. We conclude, independent of ionic strength, that the myosin heads attached to actin during the steady-state hydrolysis of ATP are in a rotationally dynamic state that is almost as mobile as the detached cross-bridges during relaxation, even within the constraints of the myofibrillar lattice.

Un-Cross-Linked Myofibrils. Tryptic digestion and ST-EPR experiments also were performed on MSL-myofibrils without EDC treatment. While these myofibrils shorten upon activation and their striations are no longer visible by phase-contrast microscopy, the steady-state acto–myosin interaction (which occurs after this initial shortening) appears to be quite similar to that of cross-linked myofibrils and isometric skinned muscle fibers: (1) The ATPase activity of myofibrils is similar to that of single isometric muscle fibers (Matta & Thomas, 1992). (2) The ST-EPR spectrum of MSL-myofibrils usually returns nearly to the rigor value following activation and subsequent depletion of ATP (data not shown). (3) The fraction of actin-attached myosin heads is almost as great in MSL-myofibrils as in EDC-MSL-myofibrils at both physiological (0.21 ± 0.04) and low (0.36 ± 0.03) ionic strengths. (4) Similar steady-state ST-EPR spectral intensity values were obtained in active un-cross-linked MSL-myofibrils at both physiological (0.52 ± 0.03) and low (0.54 ± 0.03) ionic strengths. (5) The microsecond rotational motion of actin-bound myosin heads in active MSL-myofibrils is essentially the same as that obtained with EDC-MSL-myofibrils at physiological (τ_r = 3.9 ± 1.7 μs) and low ionic strengths (τ_r = 4.2 ± 1.0 μs). The principal advantage of the cross-linked preparation is that (1) these myofibrils maintain their macroscopic structure, as viewed in an optical microscope, and (2) the biochemical and spectroscopic data obtained in the active state are more reproducible.

DISCUSSION

Summary of Results. We have used ST-EPR to measure the microsecond rotational motions of actin-attached myosin heads in active (isometrically contracting) EDC-MSL-myofibrils. During the brief steady state of ATP hydrolysis, initiated by the photolysis of caged ATP in the presence of Ca^{2+}, the microsecond rotational motion of MSL-labeled myosin heads increased dramatically, almost to the level of relaxation. The fraction of myosin heads bound to actin was determined biochemically from tryptic digestion binding experiments, permitting us to calculate the ST-EPR spectrum of the actin-bound population of myosin heads. We found that the observed increase in rotational motion upon photolysis of caged ATP was due not only to the detachment of myosin heads from actin but also to an increase in the rotational motion of the actin-bound myosin heads. The rotational mobility of the actin-attached myosin heads was indistinguishable at low and physiological ionic strengths (τ_r = 3.2–3.5 μs).

Tryptic Digestion Binding Results. The determination of the fraction of actin-bound myosin heads in active myofibrils depends on the rate of tryptic digestion of the detached myosin heads, measured in relaxed myofibrils, and of the actin-attached myosin heads 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. The relaxation conditions used (3 mM MgADP·V_i + 0.1 mM MgATP) have been shown to detach all of the myosin heads from actin in myofibrils and muscle fibers, even in the presence of Ca^{2+}. Myofibrils under these conditions are digested by trypsin at the same rate as *p*-nitrophenylenemaleimide (*p*NPM)-modified myofibrils, in which the acto–myosin affinity in the presence of ATP has been shown to be negligible (Duong & Reisler, 1989), and the active tension and stiffness of isometric muscle fibers drop to near-zero under these conditions (Goody et al., 1979). The inclusion of a small amount of ATP with ADP·V_i is necessary for relaxation since vanadate binds to a cross-bridge state that is only accessible during the active ATPase cycle (Dantzig & Goldman, 1984).

Another key assumption is that the rate of tryptic digestion of myosin heads attached to actin in the presence of nucleotide is the same as in rigor. It has been shown that the fraction of actin-bound myosin heads in solutions of acto–S1 in the presence of ATP (Duong & Reisler, 1987a), determined from the rate of tryptic digestion, agrees with sedimentation binding measurements under the same conditions. Therefore, the rigor cross-bridges are a satisfactory model for the proteolytic susceptibility of all actin-attached myosin heads, whether nucleotide is present or not. Another important consideration in the tryptic digestion binding measurements is that the steady-state distribution of actin-attached and detached cross-bridges be sampled. The rate of tryptic digestion of both the rigor and relaxed myofibrils was several orders of magnitude slower than the association and dissociation between actin and myosin under these conditions (Schoenberg, 1988, 1989), and, thus, eq 1 and 2 are valid.

Sedimentation binding experiments with solutions of HMM and actin suggest that only one head of the myosin molecule interacts strongly with actin in the presence of nucleotide (AMPPNP or ADP), presumably due to steric constraints (Duong & Reisler, 1987b). Similar results have been obtained in the presence of ADP or AMPPNP with tryptic digestion binding studies of acto–HMM in solution (Duong & Reisler, 1987b) and in myofibrils (Chen & Reisler, 1984). EPR studies on MSL-labeled muscle fibers have also shown that all (>95%) heads bind to actin in rigor (Thomas & Cooke, 1980) but AMPPNP or pyrophosphate can dissociate half of the myosin heads from actin without affecting stiffness (Fajer et al., 1988; Pate & Cooke, 1988). The small fraction (25–30%) of actin-attached myosin heads in active myofibrils, measured both in the present work and in previous studies (Duong & Reisler, 1989), compared to stiffness measurement of isometric muscle fibers (50–70%; Barnett & Thomas, 1989; Fajer et al., 1990) is consistent with cross-bridges that are attached by a single myosin head during contraction.

ST-EPR Results. The observed rotational motion in relaxed myofibrils (Figures 2 and 3) is probably representative of myosin heads detached from actin, since similar results were obtained at physiological ionic strength (μ = 165 mM) both under normal relaxation conditions (5 mM MgATP, no Ca^{2+}) and in the presence of 3 mM MgADP·V_i + 0.1 mM MgATP, conditions in which all of the myosin heads have been shown to be detached even in the presence of Ca^{2+} (Goody et al., 1979). The ST-EPR spectrum of rigor MSL-myofibrils (Figures 2–4) was quite intense, indicating that the myosin heads are rigidly bound to actin in the myofibrillar lattice. The ST-EPR intensity of active (isometrically contracting) myofibrils (Figure 4) is only slightly more than that in relaxation, despite a substantial difference in the fraction of actin-bound myosin heads (Table I: 0.00 in relaxation, 0.24 in contraction at physiological ionic strength, and 0.37 in contraction at low ionic strength). At both physiological and low ionic strengths, the results obtained with MSL-myofibrils upon the photolysis of caged ATP are consistent with the results obtained previously with acto–MSL-S1 at low ionic strength (Berger et al., 1989): *the myosin heads bound to actin in the presence of ATP under active conditions are almost as rotationally mobile as the free (or relaxed) myosin heads.* The value of τ_r is significantly longer for the actin-bound myosin heads in the myofibril (3.2–3.5 μs) than in acto–S1 (1.0 μs), probably due to (1) the restriction of motion by the myofibrillar lattice and/or (2) the different distribution of biochemical and mechanical states in the two systems. Increasing the ionic strength to physiological levels decreases the fraction of actin-attached heads (from 0.37 to 0.24) in active myofibrils, but does not significantly change the rotational motion of those attached heads (3.5 and 3.2 μs, Table I).

Relationship to EPR and ST-EPR of Muscle Fibers. The results obtained with relaxed and rigor myofibrils are consistent with previous measurements of microsecond rotational motion of myosin heads in myofibrils (Ishiwata et al., 1987; Ludescher & Thomas, 1988), and EPR of spin-labeled muscle fibers. Myosin heads in spin-labeled muscle fibers have been shown to be highly disordered (Thomas & Cooke, 1980) and rotationally mobile on the microsecond time scale (Barnett & Thomas, 1989) during relaxation, and well-ordered with respect to the thick filament axis (Thomas & Cooke, 1980) and static on the microsecond time scale (Barnett & Thomas, 1989) in the rigor state.

Previous EPR studies of un-cross-linked myofibrils in contraction (Thomas et al., 1980) or isometrically contracting muscle fibers (Cooke et al., 1982; Barnett et al., 1989; Fajer et al., 1990) also showed that MSL-labeled myosin heads are almost as orientationally disordered and mobile in contraction as in relaxation. Stiffness values in isometrically contracting muscle fibers are 70–80% of the rigor value, suggesting that most of the cross-bridges are attached to actin under these conditions (Fajer et al., 1990), a result reinforced by equatorial X-ray diffraction patterns on isometrically contracting muscle fibers, which also suggest that a majority of the cross-bridges are associated with the thin filament (Haselgrove & Huxley, 1973). Thus, a large amount of the rotational motion observed in the isometrically contracting muscle fibers was attributed to dynamically attached cross-bridges (Barnett & Thomas, 1989; Stein et al., 1990). However, neither stiffness nor X-ray diffraction measurements can determine unambiguously the fraction of actin-bound myosin heads in the isometric muscle fiber. For example, previous EPR studies have shown that nucleotides tend to induce single-headed cross-bridge binding without decreasing the stiffness per cross-bridge (Fajer et al., 1988; Pate & Cooke, 1988), so stiffness probably overestimates the fraction of heads attached to actin in contraction. Therefore, *the possibility remained that only a small, immobile population of heads are actually attached to the thin filament in active muscle* under these conditions. *This ambiguity has been resolved by the present study*, in which

the direct measurement of the fraction of bound heads has made it possible to determine the value of τ_r for the actin-bound population of myosin heads in contraction. Because the myofibrils are cross-linked, they are probably a good model for isometric muscle fibers, so *the results of the present study support the existence of a rotationally mobile population of actin-attached myosin heads in the isometrically contracting muscle fiber.*

Intepretation of Results. Conventional EPR (Fajer et al., 1990) and time-resolved phosphorescence anisotropy (TPA; Stein et al., 1990) experiments indicate that the cross-bridge population in an active isometric muscle fiber is not strictly a linear combination of relaxed and rigor cross-bridges. This interpretation is supported in the present study, in which the myosin heads have been unambiguously shown to be *dynamically attached* to actin, more mobile than in rigor but less mobile than in relaxation. The motions of actin-attached myosin heads are likely to be quite anisotropic, i.e., restricted in angular amplitude. TPA (Stein et al., 1990), which has a greater orientational resolution than ST-EPR, suggests that the amplitude of motion for myosin heads during contraction is 42° (full cone angle), a value intermediate between that of rigor (15°) and relaxation (60°). Assuming that the amplitude of restriction is 42°, simulations of ST-EPR spectra indicate that the actual rotational correlation time of the actin-bound myosin heads would be approximately 1 μs, i.e., about 3 times shorter than the effective value we have determined (Howard et al., 1993).

Conventional EPR of MSL-labeled muscle fibers, performed under the conditions of the present myofibril study, found a small but significant (12 ± 4%) population of myosin heads to be in a rigorlike orientation, and the rest highly disoriented (Fajer et al., 1990). Since the fraction of actin-attached myosin heads found in the present study is twice (24 ± 2%, Table I) the size of this oriented population, these combined results suggest that *actin-bound heads in active muscle are in at least two motional states: a relatively rigid, rigorlike state and a more rotationally mobile state.* The identification of a rotationally mobile population of attached heads does not contradict the consistent observation that the only population of heads with a well-defined orientation in contraction (or any other physiological state, for that matter) has a rigorlike orientation [Cooke et al., 1982; reviewed by Thomas (1987) and Fajer et al. (1990)].

It is plausible that cross-bridges in weakly bound states of the acto–myosin ATPase cycle are the rotationally mobile population of actin-attached myosin heads we have observed (Fajer et al., 1991) and the strong binding, force-producing states are rigidly attached to actin at a rigorlike angle. However, further studies correlating molecular dynamics with force production will be necessary to examine more directly the rotational dynamics of the actin-attached myosin heads responsible for force production. In any case, it is likely that the weakly bound intermediates of the acto–myosin ATPase cycle play an important role in the process of force generation (Huxley & Kress, 1985; Brenner et al., 1991). *Thus, the microsecond rotational motions we have observed for actin-attached myosin heads under physiological conditions in the myofibrillar lattice, whether they arise from the force-generating cross-bridges themselves, or those leading into the force-producing states of the acto–myosin ATPase cycle, are probably an integral part of the molecular mechanism of muscle contraction.*

Alternative Explanations. Our interpretation of the ST-EPR data in terms of a linear combination of signals from attached and detached heads depends on two key assumptions: (1) We assume that detached heads during the active cross-bridge cycle undergo the same rotational motion as relaxed heads. This assumption seems plausible, but in light of the evidence favoring single-headed cross-bridge attachment in active muscle (discussed above), a substantial fraction of detached heads in active muscle could be partially constrained by the attachment of their partner heads. In this case, the assumptions we have used in analyzing ST-EPR spectra would *underestimate* the mobility of the attached heads, so our conclusion that attached heads are mobile is even stronger. (2) We assume that the spectral contributions of the attached and detached populations can be analyzed independently, which amounts to assuming that they do not exchange rapidly on the ST-EPR time scale, defined by the spin–lattice relaxation time $T_1 \approx 10^{-5}$ s. If heads were to detach and reattach with rates comparable to or faster than $T_1^{-1} \approx 10^5$/s, the apparent mobility would probably be dominated by that of the more mobile (detached) heads, and we would overestimate the mobility of the immobile (attached) heads (Thomas, 1986). Recent mechanical studies on the speed dependence of stiffness do indeed indicate that active heads may attach and reattach rapidly and reversibly during isometric contraction (Brenner, 1991), but the detachment rate constants (50–1000 s^{-1}) are probably not quite high enough to affect ST-EPR spectra.

We have considered the possibility that the myosin head 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 & Thomas, 1991). Therefore, we concluded that the observed microsecond rotational motions are due to the rotation of S1 relative to actin. Another possibility is that ATP changes the conformation of myosin such that the spin-label becomes mobilized relative to the myosin head. However, while nucleotide binding to myosin has been known to mobilize certain spin-labels (Barnett & Thomas, 1987) and fluorescent probes (Thomas, 1987; Tanner et al., 1991), MSL-S1 fixed on glass beads has been shown to remain rotationally rigid on the microsecond time scale in the presence of ATP (Thomas et al., 1980). In order to determine more rigorously whether the whole myosin head is rotating and contributing to these motions, or just the 20K domain around SH_1 and SH_2, it will be necessary to perform complementary experiments with the spin-label at other sites on S1. Recent conventional EPR experiments with spin-labeled light chain 2 (LC2) exchanged into muscle fibers support the conclusion of disordered myosin heads in isometric contraction (Hambly et al., 1991a,b). Future experiments with spectroscopic probes at other sites on the myosin head will help define the role of myosin segmental flexibility in muscle contraction.

Effects of Spin-Labeling. Although the fraction of actin-bound myosin heads obtained from the tryptic digestion of active EDC-MSL-myofibrils at physiological ionic strength (0.24 ± 0.02) agrees closely with previous estimates (0.25 ± 0.07) in active EDC-myofibrils (Duong & Reisler, 1989), our measurements indicate a significant decrease in binding upon spin-labeling (10%). This is consistent with stiffness measurements on spin-labeled fibers, which suggest a similar decrease in acto–myosin binding in isometrically contracting muscle fibers (Bell et al., 1993). Spin-labeling has no effect on the acto–myosin affinity in weak binding states in solution at low ionic strength (Berger et al., 1989; Berger & Thomas,

1991), or in myofibrils in the presence of ATPγS (C. L. Berger and D. D. Thomas, unpublished results). Therefore, it is likely that spin-labeling shifts the population of cross-bridges from strong binding states to weaker binding ones. Recent experiments have shown the acto–myosin ATPase cycle is altered by SH_1 modification (Root et al., 1991; Matta & Thomas, 1992). However, even when spin-labeled up to 95% at SH_1, the spin-labeled muscle fibers can still generate a significant amount of force, indicating that the spin-labeled cross-bridges can still cycle through the entire ATPase cycle, including the strongly bound, force-producing states (Matta & Thomas, 1992; Bell et al., 1993). Thus, even the spin-labeled cross-bridges in the weakly bound states are likely to be important to the molecular mechanism of force generation, and the results obtained with spin-labeled myofibrils and muscle fibers are almost certainly applicable to unmodified muscle fibers as well.

Relationship to Other Work. We have identified a rotationally mobile cross-bridge state that is attached to actin during the myosin ATPase cycle and distinct from the predominant states in rigor or relaxation. Evidence from other techniques supports this conclusion. X-ray diffraction studies on relaxed (Yu & Brenner, 1989) and isometrically contracting (Podolsky et al., 1976; Yu & Brenner, 1987; Irving et al., 1992) skeletal muscle fibers, and on bony fish muscle (Harford et al., 1991), have also identified an actin-attached cross-bridge state that is different from the rigor state, on the basis of changes in the radial mass distribution around the thick and thin filaments. Electron microscopy of cross-linked (Craig et al., 1985; Applegate & Flicker, 1987) and non-cross-linked solutions of actin and S1 (Trinnick & White, 1991) or HMM (Frado & Craig, 1991), and of muscle fibers (Hirose et al., 1991), has shown that the myosin head is disordered while bound to actin in the presence of ATP, although another electron microscopic study reports a rigorlike orientation in the presence of ATP at 4 °C (Pollard et al., 1993). Disorder of the myosin heads while bound to actin, even in the muscle fiber, implies that myosin is quite flexible in its interaction with actin, which has been verified by electron micrographs that show myosin can form reverse-angle cross-bridges in mutant insect myofibrils with thin filaments of the wrong polarity (Reedy et al., 1989).

Previous work with EDC-cross-linked myofibrils has demonstrated that this preparation is an excellent analog of an isometrically contracting muscle fiber in solution. The steady-state ATPase activity of EDC-cross-linked myofibrils is similar to that of an isometric muscle fiber (Glyn & Sleep, 1985). Also, the rate of medium oxygen exchange via labeled phosphate, which is thought to be directly related to the amount of mechanical strain on the cross-bridge, is similar in EDC-myofibrils and isometric muscle fibers, and is much faster in these preparations than in solutions of acto–S1 (Bowater & Sleep, 1985). Since the myosin head only spends 1–2 ms at a given attachment site on actin (White & Taylor, 1976), submillisecond motions of S1 are required to move from one site of attachment on actin to another. Mechanical measurements on muscle fibers have shown that there are tension transients that follow a rapid stretch and release of the fiber during steady-state ATP hydrolysis, which probably arise from submillisecond rotations of actin-attached cross-bridges (Huxley & Simmons, 1971). The dynamic nature of active cross-bridge attachment is further underscored by the conclusion that cross-bridges 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 cross-bridge cycle.

Conclusions. We have demonstrated directly that actin-attached spin-labeled myosin heads within the myofibrillar lattice are rotationally mobile on the microsecond time scale during the active ATPase cycle at both physiological and low ionic strengths. These active attached heads are more mobile than in rigor but less mobile than in relaxation. This work extends previous measurements in solutions of acto–S1, which also directly demonstrated microsecond rotational motions of actin-attached myosin heads, but under less physiological conditions (Berger et al., 1989), and in isometric muscle fibers, in which the rotational motion of *actin-attached* cross-bridges cannot be unambiguously determined (Barnett & Thomas, 1989). Since the EDC-cross-linked myofibrils are probably a good model system for isometric muscle fibers, it is likely that the observed microsecond rotational motions during the active ATPase cycle are important to the process of force generation and myofilament sliding in the intact muscle as well.

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