EFFECTS OF AMPPNP ON THE ORIENTATION AND ROTATIONAL DYNAMICS OF SPIN-LABELED MUSCLE CROSS-BRIDGES

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ABSTRACT We have used electron paramagnetic resonance (EPR) to investigate the orientation, rotational motion, and actin-binding properties of rabbit psoas muscle cross-bridges in the presence of the nonhydrolyzable nucleotide analogue, 5′-adenylylimido-diphosphate (AMPPNP). This analogue is known to decrease muscle tension without affecting its stiffness, suggesting an attached cross-bridge state different from rigor. We spin-labeled the SH1 groups on myosin heads and performed conventional EPR to obtain high-resolution information about the orientational distribution, and saturation transfer EPR to measure microsecond rotational motion. At 4°C and 100 mM ionic strength, we find that AMPPNP increases both the orientational disorder and the microsecond rotational motion of myosin heads. However, computer analysis of digitized spectra shows that no new population of probes is observed that does not match either rigor or relaxation in both orientation and motion. At 4°C, under nearly saturating conditions of 16 mM AMPPNP (K_d = 3.0 mM, determined from competition between AMPPNP and an ADP spin label), 47.5 ± 2.5% of myosin heads are dynamically disoriented (as in relaxation) without a significant decrease in rigor stiffness, whereas the remainder are rigidly oriented as in rigor. The oriented heads correspond to actin-attached heads in a ternary complex, and the disoriented heads correspond to detached heads, as indicated by EPR experiments with spin-labeled subfragment 1 (S1) that provide independent measurements of orientation and binding. We take these findings as evidence for a single-headed cross-bridge that is as stiff as the double-headed rigor cross-bridge. The data are consistent with a model in which, in the presence of saturating AMPPNP, one head of each cross-bridge binds actin about 10 times more weakly, whereas the remaining head binds at least 10 times more strongly, than extrinsic S1. Thus, although there is no evidence for heads being attached at nonrigor angles, the attached cross-bridge differs from that of rigor. The heterogeneous behavior of heads is probably due to steric effects of the filament lattice.

INTRODUCTION

In most current molecular models of muscle contraction, force is generated by a conformational change within myosin cross-bridges after their attachment to actin, coupled to the ATPase cycle. The cross-bridge is defined as the heavy meromyosin (HMM) portion of myosin, containing two globular myosin heads (S1) linked to the thick filament backbone through a single fibrous S2 segment. This conformational change must produce strain that is relieved by the sliding of filaments past each other, shortening the sarcomere. A major objective in muscle biophysics is to detect and define this conformational change, which has been proposed to involve a change in orientation of actin-attached myosin heads with respect to the fiber axis (Huxley, 1969; Huxley and Simmons, 1971).

Of the various physical techniques that have been employed to detect myosin head rotations (reviewed by Cooke, 1986, and Thomas, 1987), an essential approach is to monitor the orientation of probes on myosin heads during the dynamic steady-state of force generation and to compare the results with those obtained in static states that might be similar to intermediates in the force-generation process. The most commonly studied static states are relaxation and rigor. It is likely that relaxation (in which the affinity of myosin for actin is extremely weak) is a good model for the detached phase of the cross-bridge cycle, whereas rigor is similar to the attached cross-bridge state that occurs at the end of the cycle. Because force generation requires a change during the attached phase of the cycle, attention has been focused on a search for an attached cross-bridge intermediate with a conformation different from rigor. However, attempts to resolve such a conformation during contraction have been complicated by the heterogeneous steady-state distribution of states and the difficulty in determining whether a particular signal component corresponds to attached or detached cross-bridges. There is also the possibility of a short-lived intermediate conformation not detected in the steady state. One means of overcoming this complexity is to perform transient experiments that can resolve different conformations in time. Another way is to use nonhydrolyzable

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nucleotide analogues producing conformations that may correspond to important intermediates in the cycle. For example, 5′-adenylylimido-diphosphate (AMPPNP) (Yount et al., 1971) has been shown to produce values intermediate between rigor and relaxation for actin–myosin affinity, tension, sarcomere length (Marston et al., 1976 and 1979; Kuhn, 1973, 1978*a* and *b*), and x-ray diffraction intensities (Lymn, 1975; Marston et al., 1976; Padron and Huxley, 1984). Because some of these changes are detectable even when stiffness is not affected, they probably reflect the properties of attached cross-bridges and may correspond to a partial reversal of the force-generating cross-bridge stroke from rigor. To study cross-bridge states produced by AMPPNP, we have used an EPR spectroscopic probe technique that is sensitive to the orientation and motion of myosin heads and offers sufficient resolution to detect and characterize any heterogeneity (reviewed by Thomas, 1987).

Previous EPR studies on glycerinated fibers and myofibrils, using a maleimide spin label that reports the overall orientation and motion of the myosin head, have shown that all myosin heads are attached to actin and uniformly oriented in rigor, but are dynamically disordered when dissociated from actin by relaxation (Thomas et al., 1980; Thomas and Cooke, 1980) or fiber stretching (Barnett and Thomas, 1984). 5 mM AMPPNP produces a heterogeneous orientational distribution, yielding two principal orientational components that are similar to those observed in rigor and relaxation (Thomas and Cooke, 1980). Those preliminary results in the presence of AMPPNP are particularly intriguing, because the apparently bimodal distribution observed is qualitatively similar to the one observed during isometric contraction (Cooke et al., 1982).

In the present study, we have extended the previous AMPPNP studies to measure both the orientation (by conventional electron paramagnetic resonance [EPR]) and rotational motion (by [ST]-EPR) of the same preparation of spin-labeled fibers in rigor, relaxation, and various concentrations of AMPPNP, including nearly saturating levels. We have performed a quantitative computer analysis to resolve and characterize the spectral components to determine whether AMPPNP produces any new orientations or motions not observed in rigor or relaxation. Stiffness measurements on the same fiber preparations provided information about cross-bridge attachment, and EPR spectra of labeled S1 diffused into unlabeled fibers have been used to determine whether specific spectral components arise from attached or detached heads and to determine the effects of the fiber lattice on the affinities of actin and nucleotides for myosin heads. Complementary results on the effects of pyrophosphate (PP_i) on spin-labeled fibers are reported in the accompanying paper by Pate and Cooke (1988).

METHODS

Sample Preparation

New Zealand white rabbits (~4 kg) were killed by CO_2 asphyxiation or by anesthetization with 75 mg ketamine hydrochloride per kg weight, bled, and small strips of psoas muscle (~2 mm diameter) were removed. The fiber bundles were incubated at 4°C on a shaker in fiber glycerination (FG) solution (solution compositions in Table I) for 24 h, then transferred to fiber storage (FS) solution for 48 h before ultimate storage at −20°C. Fibers were stored for a maximum of 3 mo.

Subfragment 1 (S1) was prepared from myosin by digestion with α-chymotrypsin in 120 mM KCl, 10 mM morpholino propane sulfonic acid (MOPS), 1 mM EDTA, essentially as described by Weeds and Taylor (1975), retaining the fraction soluble in 48% saturated ammonium sulfate but not in 60%. The fraction of S1 molecules capable of binding to actin was measured by incubation with an excess of actin or myofibrils in rigor buffer for 20 min at 24°C, followed by sedimentation of 150,000 g for 40 min. The concentration of S1 in the supernatant, determined from absorption at 280 nm or from EPR, was always <5% of the total S1. All binding constants were corrected for this nonbinding fraction.

Protein Concentration

The concentration of protein in myofibril suspensions was determined by the biuret assay, using bovine serum albumin as a standard. The concentration of purified S1 was determined from the expression [S1, mg/ml] = [A(280 nm) − A(320 nm)]/0.75 (Margossian and Lowey, 1982).

ATPase Assays

The ATPase activity in the presence of saturating ATP was determined by measuring inorganic phosphate (P_i) production. The reaction was

TABLE I
SOLUTIONS

Solution	KAc	KPr	$MgCl_2$	EGTA	MOPS	ATP	$CaCl_2$	Glycerol	NaN_3	Triton
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*%*	*mM*	*%*
FG	60	—	2	6	25	—	—	25	1	0
FS	60	—	2	6	25	—	—	50	1	—
Rigor	—	130	2	1	20	—	—	—	1	—
Relaxation	—	130	7	1	20	5	—	—	1	—
AMPPNP (16 mM)	—	6	18	1	10	—	—	—	—	—

All solutions were adjusted to pH = 7.0 at 4°C. Solutions containing other concentrations of Mg AMPPNP were prepared by substituting 5 mM KPr per Mg AMPPNP to keep ionic strength at 100 mM. For experiments at μ = 220 mM, 120 mM KPr was added to the corresponding μ = 100 mM solution. +Ca solutions were obtained by adding 1 mM $CaCl_2$ to the −Ca solution, as given above.

initiated by the addition of 5 mM MgATP, and aliquots were quenched at timed intervals in an acidic P_i-determining solution (Lanzetta et al., 1979) and then assayed by measuring the absorbance at 660 nm. Fibers were assayed as myofibril suspensions.

Physiological ATPases were measured in a relaxing solution ± 0.5 mM Ca^{2+} at 25°C, and at a protein concentration of 0.2–0.4 mg/ml. High-salt ATPase activity was measured in 0.6 M KCl, 50 mM MOPS, pH 7.5, 25°C, with the addition of 10 mM EDTA for the K/EDTA-ATPase or 10 mM $CaCl_2$ for the Ca/K-ATPase, at a protein concentration of ~0.3 mg/ml for myofibrils and ~0.1 mg/ml for S1. The fraction of the fast-reacting thiol (SH1) groups labeled was estimated from the fractional inhibition of the K/EDTA-ATPase activity (Crowder and Cooke, 1984).

Spin Labeling

Glycerinated rabbit psoas fibers were dissected in RB buffer into 0.5-mm bundles, and then labeled with 0.25 mM MSL *N*-(1-oxyl-2,2,2,6-tetramethyl-4-piperidinyl)maleimide (maleimide spin label, Aldrich Chemical Co., Milwaukee, WI) in rigor buffer plus 4 mM $MgPP_i$ for 20 min at pH 6.5 (Crowder and Cooke, 1984). Fibers were then treated with 25 mM $K_3Fe(CN)_6$, 5 mM cysteine, 1 mM EDTA, 10 mM MOPS, pH 7.0, under nitrogen, for 0–14 h. Typically 50–70% of the SH1 thiol groups of myosin were labeled by MSL, as estimated from the fractional inhibition of K/EDTA-ATPase. Physiological ATPases were not significantly affected by labeling or by 5 h $K_3Fe(CN)_6$ oxidation. Ca^{2+}-ATPase values were 0.273, 0.237, and 0.265 IU (μmol P_i/mg protein per min) for control, MSL-labeled, and oxidized fibers, respectively. The specificity of labeling, as estimated from the fraction of oriented component, was 20–40% of the total signal. This specificity increased to 40–70% after ferricyanide treatment. Purified S1 was labeled with MSL (Svensson and Thomas, 1986). The label was predominantly attached to SH1, because the fractional ATPase inhibition (0.85–1.0) was in good agreement with the labeling stoichiometry determined from double integration of EPR spectra (Thomas et al., 1980). ADP spin label (2′-3′-0-[1-oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine] adenosine 5′-triphosphate) used in the determination of the association constant for AMPPNP was a gift from Dr. R. Cooke and Dr. M. Crowder. The AMPPNP used was the tetralithium salt, purchased from Sigma Chemical Co., St. Louis, MO. ^{31}P-NMR showed that the triphosphates present were <1.7%.

Mechanical Measurements

The tension generated by single fibers was measured using an Ackers 801 strain gauge (Aksjelskapet, Norway). The active tension at 25°C ranged from 2 to 3 kg/cm^2 before labeling. The active tension relative to the unlabeled control was 88 ± 4% after labeling, 74 ± 7% after 5 h of $K_3Fe(CN)_6$, and 42 ± 10% after 14 h of $K_3Fe(CN)_6$. For preparations used to obtain EPR data, resting tension was no more than 3% of the control active tension, although some $K_3Fe(CN)_6$-treated samples had values as high as 10%. Stiffness measurements were performed by Dr. Cooke and are expressed as a fraction of the rigor stiffness; for details see the companion paper of Pate and Cooke, 1988. Spin labeling and 4 h of $K_3Fe(CN)_6$ treatment had no effect on the fractional stiffness in relaxation and AMPPNP.

Electron Paramagnetic Resonance

0.5-mm fiber bundles were placed in 1-mm glass capillaries and held isometrically by surgical silk thread tied to the bundle ends. Solution was continuously flowed over the fibers at a rate of 0.2 ml/min. EPR experiments were performed on a Bruker ER-200D spectrometer (IBM Instruments Inc., Danbury, CT) using a TM_{101} cavity modified to accept a fiber-containing capillary parallel to the static magnetic field (Thomas and Cooke, 1980). Conventional EPR spectra were recorded at a microwave field (H_1) of 0.06 G and a modulation field (H_m) of 2 G. ST-EPR (V'_2) spectra were recorded at H_1 = 0.2 G and H_m = 5 G as calculated from cavity Q and peroxylamine disulfonate calibration (Fajer and Marsh, 1982; Squier and Thomas, 1986). Temperature was controlled by flowing precooled N_2 gas through the radiation slits into the cavity or by placing the cavity in a thermally insulated box in which the temperature was regulated using a Bruker VT-100 temperature controller. Spectra were acquired, averaged, and analyzed using a Zenith 158 microcomputer (Zenith Data Systems, St. Joseph, MI) and a Data Translation 8021A A/D board (Data Translation Inc., Marlboro, MA), using software developed by the authors.

Spectral Analysis

Each EPR spectrum of fibers in AMPPNP was compared with composite spectra that were created by the addition of various mole fractions of the spectra obtained from the same fiber bundle in rigor and relaxation buffers. A library of such composite spectra was compiled, and the experimental spectrum was subtracted from each composite spectrum. Minimization of the residual was used to determine whether the experimental line-shape could be expressed as a linear combination of rigor and relaxed spectra, and, if so, to determine the mole fraction of each component. This method of analysis was successfully applied for both conventional EPR and ST-EPR spectra.

RESULTS

ATP Induces Dynamic Disorder in All Heads

The conventional EPR spectrum of a maleimide spin-labeled muscle fiber in rigor oriented parallel to the external magnetic field (Fig. 1 *a*) has three sharp peaks, characteristic of a highly oriented population of probes,

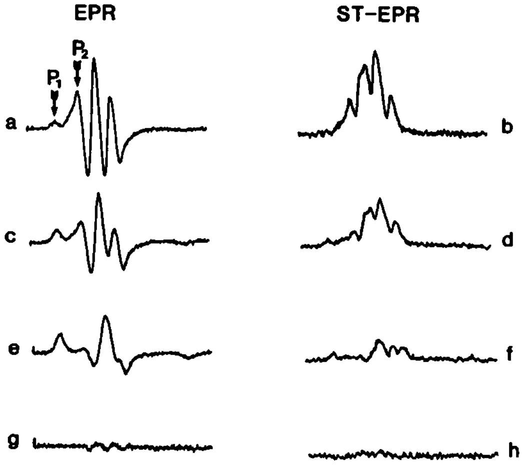

FIGURE 1 EPR spectra of rabbit psoas muscle fibers labeled with maleimide spin label and oriented parallel to the external magnetic field. (*Left*) Conventional EPR, (V_1: first harmonic, in-phase absorption) spectra. (*Right*) Saturation transfer (V'_2: second harmonic, out-of-phase absorption) spectra. The first three rows show the spectra in rigor, 16 mM AMPPNP, and relaxation (5 mM ATP), respectively (see Table I for solution composition). The bottom row displays the residual spectrum obtained by subtracting the best-fit composite spectrum from the AMPPNP spectrum (see Methods). The composite spectrum (not shown) was created by a digital addition of 52.5% of the rigor spectrum and 47.5% of the relaxation spectrum, for both columns. Arrows illustrate the spectral positions where P_2, characteristic of a narrow orientational distribution, and P_1, characteristic of a highly disoriented distribution, are measured as peak heights above the baseline. All spectra were recorded at 4°C. The baseline in these and other spectra shown below is 100 G wide.

with a mean orientation of ~80° and a narrow orientational distribution (Thomas and Cooke, 1980; Barnett et al., 1986). There is little or no microsecond motion, as indicated by the high intensity of the ST-EPR spectrum (Fig. 1 *b*). Relaxation by ATP produces an increase in intensity at the P_1 position at the expense of the intensity at P_2, which indicates significant disorientation (Fig. 1 *e*). The decreased intensity of the V_2' spectrum (Fig. 1 *f*) implies that the disorientation is accompanied by increased submillisecond rotational motion, i.e., dynamic disorder (Barnett and Thomas, 1984; Evans, 1981).

Conventional EPR spectra of randomly oriented myofibrils obtained from these fibers was unaffected by ATP or AMPPNP (data not shown), implying that all of the nucleotide effects on conventional EPR spectra in Fig. 1 are due to changes in the orientational distribution (not to changes in nanosecond motions), and that all of the effects on ST-EPR spectra are due to changes in microsecond rotational motions. This is consistent with the lack of significant ATP effects on conventional EPR spectra of MSL-labeled myosin filaments (Barnett and Thomas, 1984) or fibers stretched to nonoverlap (Fajer et al., 1985). The lack of nanosecond motions strongly suggests that the nucleotide-induced motions do not correspond to librational motion of the spin label or a localized binding domain, but rather correspond to large-scale protein rotations, probably of the entire myosin head. The conclusion that these spectra reflect the overall rotation of heads is reinforced by previous ST-EPR studies. (*a*) When cross-linked to glass beads, S1 is much less mobile than in myosin filaments or relaxed myofibrils, and ATP effects are negligible (Thomas et al., 1980), and (*b*) ST-EPR spectra of MSL-labeled myosin filaments are unaffected by ATP or AMPPNP (Thomas et al., 1980; Ishiwata et al., 1986). Thus in the following we will use "myosin head orientation" or motion when discussing the orientation or motion of the spin label. In rigor (Fig. 1, *a* and *b*) the observed uniform orientation and immobilization, and the high stiffness (Table II) are consistent with all the myosin heads being rigidly attached to actin filaments (Thomas et al., 1980; Thomas and Cooke, 1980). Similarly, in relaxation (Fig. 1, *e* and *f*) the dynamic disorientation (similar to that observed in filaments of purified myosin) and negligible stiffness (Table II) is consistent with the myosin heads being detached from actin and executing nearly free Brownian motion.

AMPPNP Induces Dynamic Disorder in Half of the Heads

The addition of 16 mM AMPPNP (μ = 100 mM) to rigor fibers results in spectra intermediate between those of rigor and relaxation (Fig., 1 *c* and *d*).

Spectral deconvolution of the V_1 and V_2' line-shapes (see Methods) yields negligible residuals for a 0.475:0.525 mixture of relaxed and rigor spectra (Fig. 1, *g* and *h*), strongly suggesting that the orientational distribution of myosin heads is a linear combination of those in rigor and relaxation despite the fiber stiffness remaining at the rigor value (Table II). It is particularly important to know whether the highly oriented heads in the presence of AMPPNP (giving rise to the sharp lines in Fig. 1 *c*) differ

TABLE II

SUMMARY OF FIBER EPR AND STIFFNESS DATA

Conditions	% Oriented*	% Immobile†	% Stiffness§
Rigor	=100	=100	=100
Relaxation (5 mM ATP)	= 0	= 0	5 ± 5 (6)
4 mM AMPPNP, μ = 100 mM			
−Ca	68 ± 3 (6)	68 ± 5 (4)	100 ± 10 (3)
+Ca	82 ± 4 (2)	80 ± 5 (2)	—
4 mM AMPPNP, μ = 220 mM			
−Ca	57 (1)	55 (1)	92 (1)
+Ca	69 (1)	67 (1)	—
16 mM AMPPNP, μ = 100 mM	52 ± 3 (6)	52 ± 4 (6)	100 (5)
50% Glycol‖	66 (1)	—	39 (1)
4 mM AMPPNP + 50% glycol			
−Ca	0 ± 1 (3)	0 ± 0 (2)	16 (1)
+Ca	0 (1)	0 (1)	—
5 mM ATP + 50% glycol	= 0	= 0	—
4 mM PP_i	58 (1)	57 (1)	100 (1)

All data were obtained at 4°C and pH 7.0. All were obtained at a final ionic strength of 170 mM, unless otherwise noted. EPR spectra were analyzed as a linear combination of those recorded in rigor and relaxation, as demonstrated and justified in Fig. 1. Unless otherwise noted, all solutions contained 1 mM EGTA and no $CaCl_2$ ("−Ca"). Those designated "+Ca" contained an additional 1 mM $CaCl_2$. Solutions were as described in Table II. The number of determinations, *n*, is given in parentheses. The uncertainty is the SEM for $n > 2$, and half the range for $n = 2$. *From conventional EPR spectra, taken as 100% in rigor, 0% in relaxation. †From ST-EPR spectra, taken as 100% in rigor, 0% in relaxation. §Stiffness was taken as 100% in rigor. ‖Due to nonspecific effects of glycol on EPR spectra, the disordered fraction for conventional EPR was obtained by subtraction of the spectrum in glycol plus ATP, until an endpoint with no disordered peak (P_1) was reached. A clear endpoint was observed in conventional EPR, but the lack of a clear endpoint in ST-EPR spectra prevented quantitative analysis.

at all in orientation from those in rigor. Therefore, the difference spectrum (obtained by subtracting 47.5% of the relaxed spectrum from the AMPPNP spectrum) is compared with the rigor spectrum (Fig. 2, *dotted curve*), for both conventional EPR and ST-EPR in Fig. 2. In the conventional EPR spectrum (Fig. 2, *top*), the splitting between the three lines differs by no more than 0.1 G, indicating that the principal axis of the spin label rotated by no more than 0.5°. The position of the center of the spectrum has changed by no more than 0.1 G, implying that the minor axes of the spin label have rotated no more than 1.5°. The line widths differ by no more than 0.3 G, implying that the width of the angular distribution differs by no more than 6° (Barnett et al., 1986). Thus the only population of heads having a well-defined axial orientation has not rotated significantly in response to AMPPNP. Similarly, the excellent agreement of the ST-EPR spectra (Fig. 2, *bottom*) indicates that this fraction of heads is just as rigidly immobilized as in rigor. Similar results were obtained at 4 mM AMPPNP except that the fraction remaining oriented was 0.35 ± 0.02 (SEM).

The data in Fig. 1 were obtained from a fiber bundle treated for 14 h in $K_3Fe(CN)_6$. Fibers treated for shorter times, or not at all, with $K_3Fe(CN)_6$ had a larger spectral contribution from nonspecifically bound label (resulting in a greater intensity at P_1), but the conclusions were the same as those obtained from Figs. 1 and 2. The AMPPNP spectra were well fit by an approximately 50:50 composite of rigor and relaxation spectra (Thomas et al., 1988).

Nucleotide Binding

In light of the heterogeneous population of heads detected in the presence of 16 mM AMPPNP, it is essential to determine what fraction of heads has bound nucleotide.

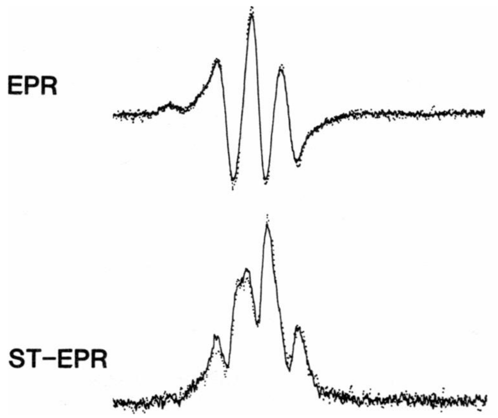

FIGURE 2 Comparison of the oriented spectral component observed in the presence of AMPPNP (*dotted line*) with the spectrum observed in rigor (*solid line*, same as first row of Fig. 1). (*Top*) Conventional EPR. (*Bottom*) ST-EPR. The solid curves were obtained by taking the mole fraction (47.5%) determined for the disoriented component in Fig. 1, and subtracting that fraction of the relaxation spectrum (third row of Fig. 1) from the AMPPNP spectrum (second row of Fig. 1), then normalizing the difference and rigor spectra to the same number of spins.

We interpret the binding of actin (A) and nucleotide ligands (L) to myosin heads (M) according to:

$$\begin{tikzcd} M \arrow[r, shift left, "K_1"] \arrow[d, shift left] & M \cdot L \arrow[l, shift left] \arrow[d, shift left] \\ A \cdot M \arrow[u, shift left, "K_2"] \arrow[r, shift left] & A \cdot M \cdot L \arrow[u, shift left, "K_3"'] \arrow[l, shift left, "K_4"] \end{tikzcd}$$

SCHEME I

The binding constants shown are all association constants. It has been shown previously that $K_1 = 1.8 \times 10^6$ M^{-1} for free S1 and AMPPNP (Wagner and Yount, 1975), so that essentially all free myosin heads must have bound AMPPNP in Fig. 1, *c* and *d*.

To determine the fractional saturation of the actin-bound myosin heads with AMPPNP, the binding constant K_4 was determined by competitive binding with spin-labeled ADP (ADPSL). The peaks corresponding to bound and freely tumbling ADPSL species can be resolved in the spectra, permitting quantitative measurement of their concentrations (Crowder and Cooke, 1987). The inhibition constant K_4 for AMPPNP was calculated:

$$B = \frac{B_{max}[\mathrm{ADPSL}]}{K_d(1 + [\mathrm{AMPPNP}]K_4) + [\mathrm{ADPSL}]} \tag{1}$$

where B is the fraction of sites occupied by ADPSL and K_d is the dissociation constant K_4^{-1} for L = ADP in Scheme I. K_d was first determined from binding experiments with ADPSL alone (Fig. 3). A least-squares fit of the data to the single-site binding isotherm ($B = B_{max}[\mathrm{ADPSL}]/(K_d + [\mathrm{ADPSL}])$) gave K_d = 152 ± 70 μM and the maximum number of binding sites B_{max} = 236 ± 88 μM. These figures agree well with K_d = 140 ± 70 μM for ADPSL (Crowder and Cooke, 1987), and a myosin head concentration in a fiber of 240 μM (Tregear and Squire, 1973), confirming that ADPSL binds specifically to the active site of myosin (Crowder and Cooke, 1987). The addition of 4 mM AMPPNP to a fiber in the presence of 150 μM ADPSL decreased the bound fraction of ADPSL from 100 to 45%. Substitution of these values into Eq. 1 yields a value of $K_4 = 330 \pm 140$ M^{-1} for AMPPNP. Therefore, the fraction of actin-bound heads that have bound AMPPNP is 84 ± 6% at 16 mM AMPPNP, and 53 ± 4% at 4 mM AMPPNP.

S1 Binding

The simplest interpretation of the data in Fig. 1 is that dynamically disoriented heads are detached from actin, but further data are required to rule out the possibility that the heads are attached. Therefore, to obtain simultaneous information about both orientation and attachment, experiments were performed on MSL-S1 diffused into unlabeled fibers (Fig. 4). The fiber bundle, in a 20-μl capillary, was

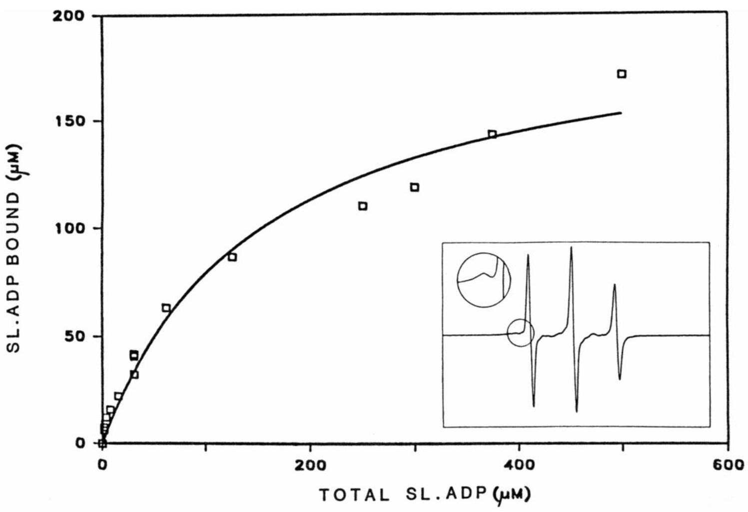

FIGURE 3 Binding of ADP spin label to rabbit psoas fibers. The data were fitted using nonlinear least square methods to a curve describing binding to B_{max} = 236 µM of equivalent sites with a dissociation constant K_d = 152 µM. The concentration of bound label was determined from the low-field peak of the immobilized component of the EPR spectrum (*inset*) corrected for the contribution of the free peak (Crowder and Cooke, 1987).

maintained at equilibrium with a 5-ml reservoir of 80 µM MSL-S1. Thus any change in the total intensity of the EPR signal must arise from a change in the concentration of bound S1. In the absence of nucleotide, at both 4 and 18°C, two components are observed in the spectrum (Fig. 4, *top*). The peak at P_1 arises from free S1 in solution in the interfilament and interfiber spaces (thus randomly oriented). The peak at P_2 arises from S1 bound to actin (thus oriented as intrinsic heads, as shown by Thomas and Cooke, 1980). When ATP is added under relaxing conditions, the intensity at P_2 is reduced to zero (Fig. 4, *bottom*), implying the absence of rigorlike bound S1. As in the case of intrinsically labeled heads, no new spectral component is observed, implying that no new orientation is induced. In addition, there is no increase in the intensity at P_1, implying that P_1 can be accounted for entirely by free (actin-detached) S1.

In the presence of 16 mM AMPPNP at 4°C, there is a small but significant contribution to the peak at P_2 (Fig. 4 *b*), implying that a small fraction (10 ± 5%) of the S1 heads are attached with a rigorlike orientation. In fact, the difference spectrum corresponding to the oriented heads (obtained essentially as in Fig. 2, *top*) in the presence of AMPPNP matches the rigor spectrum quite well (Fig. 5, *left*), implying that the mean axial probe orientation differs from that of rigor by no more than 0.5°. However, there is still no increase in intensity at P_1, implying that there are no attached but disoriented heads. Essentially the same results were obtained at 18°C (Fig. 4, *right;* Fig. 5, *right*) except that the fraction oriented in 16 mM AMPPNP is 62% of that in rigor. Thus at both 4 and 18°C all bound S1 molecules have their spin labels oriented as in rigor, whether AMPPNP is bound to S1 or not. This strongly suggests that all actin-bound intrinsic heads (Fig. 1) have rigorlike orientations even in the ternary complex with MgAMPPNP, and that the spectral component due to disoriented probes in Fig. 1 corresponds to heads detached from actin.

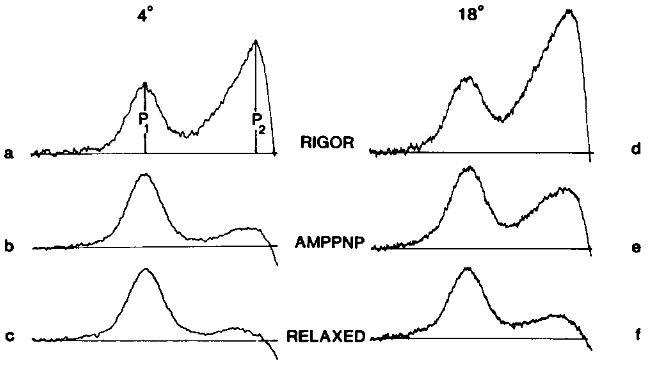

FIGURE 4 Conventional EPR spectra (low-field region) of MSL-labeled S1 binding to an unlabeled fiber bundle oriented parallel to the magnetic field. (*Top*) Rigor. (*Center*) 16 mM AMPPNP. (*Bottom*) Relaxation (5 mM MgATP added to the AMPPNP buffer). (*Left*) 4°C. (*Right*) 18°C. The S1 concentration was 80 µM. The baseline of each spectrum is 30 G. P_1 and P_2, defined as in Fig. 1, arise from highly disoriented and highly oriented (rigorlike) probes, respectively.

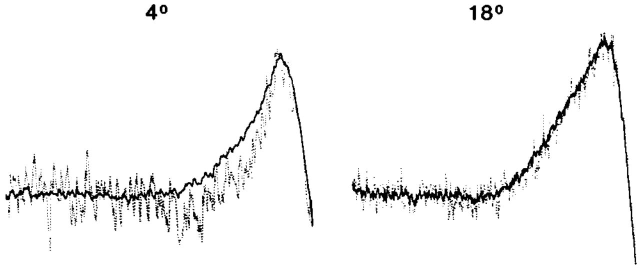

FIGURE 5 Comparison of the oriented spectral component from the conventional EPR spectrum of MSL-S1 (from Fig. 4) observed in the presence of AMPPNP (*dotted line*) with the spectrum observed in rigor (*solid line*, same as first row of Fig. 4). (*Left*) 4°C. (*Right*) 18°C. The difference spectrum (*dotted*) was obtained by subtracting the ATP spectrum (Fig. 4, *c* and *f*) from the AMPPNP spectrum (Fig. 4, *b* and *e*), until the disoriented spectral component was removed (i.e., P_1 was reduced to 0), and then normalizing the spectra to the same number of spins. The mole fraction of oriented probes determined from this procedure was 0.085 ± 0.015 (mean ± SEM for n = 3) at 4°C and 0.62 ± 0.03 (n = 1) at 18°C.

Having shown that the EPR signals provide a direct measurement of the fraction of bound S1, we can use these data to determine the affinity constant K_3 for the binding of extrinsic S1.L to A (see Scheme I). The apparent association constant K_{app} for S1 binding to actin in fibers is defined as $K_{app} = [S1 \cdot L_{bound}]/[S1 \cdot L_{free}]*[A]$, where [A] is the concentration of free actin. $[S1_{free}]$ is determined by the reservoir concentration [S1].

Because the free S1 makes no contribution to P_2, $[S1_{bound}]$ is proportional to P_2. Therefore, we can determine K_{app} empirically from:

$$K_{app} = \frac{1}{[S1]} \frac{P_2^{AMPPNP}}{P_2^{rigor} - P_2^{AMPPNP}}. \tag{2}$$

We have not corrected for the increase in concentration of actin due to dissociation of the intrinsic heads. Such a correction would be rather difficult as we cannot say whether the actin monomers that were binding the intrinsic heads in rigor and became free in AMPPNP are capable for steric reasons of binding extrinsic S1. Thus our estimate of K_{app} is an upper bound and we could have overestimated it by as much as 18% if half of the actins occupied by the intrinsic heads in rigor are binding S1 in the presence of ligand.

The relationship of K_{app} to the other binding constants in Scheme I is given by Greene and Eisenberg (1978).

$$K_{app} = \frac{K_3}{K_4[L]} + K_3, \quad \text{so } K_3 = K_{app}K_4[L]/(1 + K_4[L]). \tag{3}$$

We assume that K_4 determined above for AMPPNP binding to the actin-attached intrinsic myosin head is the same as for extrinsic S1 bound to actin in the fiber. Then determining K_{app} from our S1 EPR data and substituting into Eq. 3, we obtain K_3 (for S1 · AMPPNP binding to actin in the fiber) = 5,000 ± 2,000 M^{-1} for 4 mM AMPPNP and 900 ± 400 M^{-1} for 16 mM AMPPNP.

[AMPPNP] Dependence of Intrinsic Cross-Bridge Binding in Fibers

The affinity of intrinsic myosin heads for actin in a fiber can be estimated from the dependence of EPR spectra on [AMPPNP]. The fraction of disoriented heads, determined by deconvolution as described in Methods (and demonstrated in Fig. 1), is plotted as a function of [AMPPNP] in Fig. 6. Assuming that the disoriented fraction corresponds to ML (detached heads) and the oriented population corresponds to AM + AML, as shown above, the titration curve can be described in general by:

$$f = \sum_{i=1}^{n} \frac{x_i K_4[L]}{K_{3i} + K_4(K_{3i} + 1)\,[L]}, \tag{4}$$

where f is the fraction of disoriented heads and $K_{3i} = K_3 * A_i^{eff}$ is the ternary complex binding constant. We have allowed for n different populations of myosin heads, each having a different value of K_{3i}, defining the effective actin concentration A_i^{eff}. The molar fraction of each population is denoted by x_i. K_3 is defined as the value obtained for free S1 binding in a fiber. As discussed above, this value

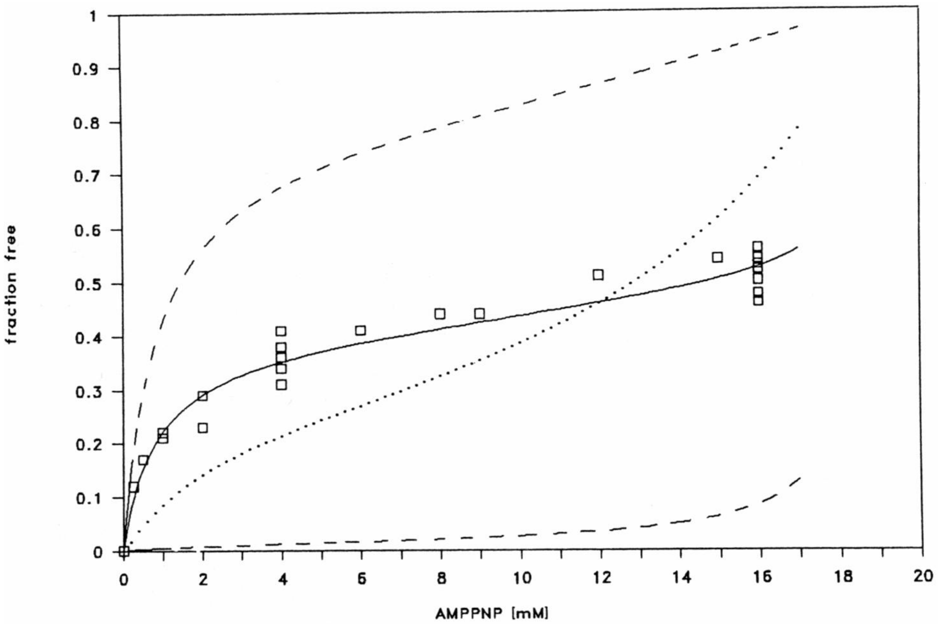

FIGURE 6 AMPPNP titration of the disoriented fraction measured from the conventional EPR spectrum of MSL-labeled intrinsic heads in fibers. The fraction free is defined as the fraction disoriented, determined as described in Fig. 1. The dotted line is the best fit for a single population of myosin heads (A^{eff} = 1.2 mM), the solid line is the best fit according to Eq. 4 for two myosin head populations as described in the text, and the dashed lines are the weak (*top*) and strong binding (*bottom*) head populations for this two-population fit (A_1^{eff} = 0.055 mM, x_1 = 0.525, and A_2^{eff} = 10 mM). Each curve was normalized to a maximum value of 1. Changes in [AMPPNP] were compensated for with KPr to maintain μ = 100 mM, and the temperature was 4°C.

depends on [AMPPNP], presumably because of its dependence of the fraction of actin sites occupied by heads in the regulated thin filaments (Greene and Eisenberg, 1980). We have assumed that K_3, to a first approximation, depends linearly on [AMPPNP]. Therefore, based on the values obtained at 4 and 16 mM AMPPNP, $K_3 = 6{,}370 - 34{,}200$ [AMPPNP]. Other plausible expressions for K_3 do not significantly change the following conclusions.

The data were first fit to Eq. 4 for a single population of heads ($n = 1$) by varying A^{eff} and minimizing the residual between fit and experiment. The best fit is plotted as a dotted curve in Fig. 6, which shows that a single population is a very poor fit. Therefore, we assumed two populations of heads (two values of A^{eff}), and varied the parameters A_1^{eff}, A_2^{eff}, and x_1 to obtain the best fit to the data. This procedure was simplified by assuming that no more than 10% of the cross-bridges have both of their heads dissociated from actin at 16 mM AMPPNP, as suggested by rigorlike stiffness in AMPPNP (Table II). This effectively requires that the stronger binding of the two head populations (having the higher A^{eff}) has no more than 10% heads dissociated at 16 mM AMPPNP. Thus we can fix A_2^{eff} at any value higher than 5 mM (we used 10 mM, which gave slightly better fits than 5 mM), and vary only A_1^{eff} and x_1. The ranges of these parameters producing satisfactory fits were $A_1^{\mathrm{eff}} = 0.55 \pm 0.015$ mM and $x_1 = 0.525 \pm 0.05$.

Effects of Glycol

Complete disorientation of intrinsic myosin heads was achieved upon the addition of 50% glycol to the solution containing 4 mM AMPPNP (Fig. 7 *e* and *f*, Table II). Both conventional (V_1) and saturation transfer (V_2') EPR spectra are indistinguishable from the corresponding spectra when ATP is substituted for AMPPNP in presence of glycol (Fig 7 *g* and *h*), and the stiffness is very low for both (Table II). The presence of 50% glycol alone increases the intensity of P_1 with a concomitant decrease at P_2, indicative of some disorientation (probably detachment) of the myosin heads (Fig. 7 *c*). Glycol has a nonspecific effect on the EPR spectra (due to changes in viscosity and dielectric constant), so the spectra obtained in relaxation are different (especially for ST-EPR) from those in the absence of glycol.

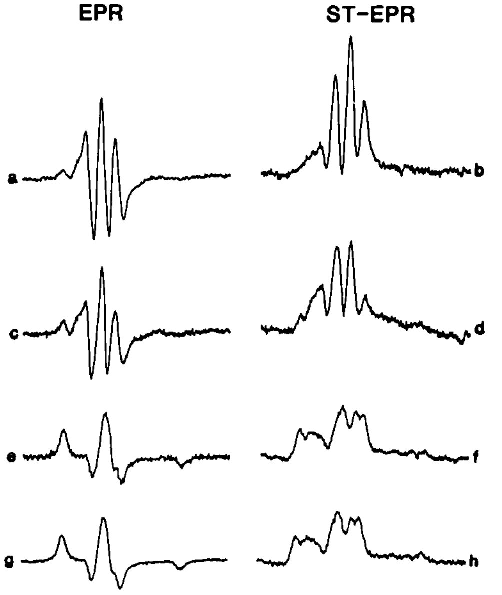

FIGURE 7 The effect of 50% ethylene glycol on the conventional EPR (*left*) and ST-EPR spectra (*right*) of MSL-labeled psoas muscle fibers. (*a* and *b*) Rigor. (*c* and *d*) rigor plus 50% glycol. (*e* and *f*) 4 mM AMPPNP and 50% glycol. (*g* and *h*) Relaxation (5 mM ATP) plus 50% glycol. Solutions are defined as in Table I, except that 50% glycol was substituted for water. $\mu = 100$ mM; T = 4°C.

Effects of Ca^{2+}

The addition of 1 mM $CaCl_2$ (producing 10 μM Ca^{2+}) to the solution containing 4 mM AMPPNP partially reversed the effect of the nucleotide. At $\mu = 100$ mM and at $\mu = 220$ mM, calcium significantly decreased the fraction of dynamically disoriented heads (Table II), indicating an increased affinity of heads for actin.

Effects of PP_i

Pyrophosphate (PP_i) is another ATP analogue that is known to cause partial relaxation of rigor fibers (see Pate and Cooke, 1988). Its effect on EPR spectra, both V_1 and V_2', was slightly greater than that of AMPPNP at the same concentration, but the same agreement between V_1 and V_2' spectra was observed, and a substantial fraction of heads was dynamically disordered without a significant change in stiffness (Table II).

DISCUSSION

Summary of Results

Our primary goal in this study was to characterize the orientation and rotational motion of heads having AMPPNP bound to them in muscle fiber cross-bridges. Of special interest was to determine whether AMPPNP induces a conformation in actin-attached cross-bridges that is orientationally and/or dynamically distinguishable from rigor. In addition to answering this question, the combination of EPR data with stiffness and binding measurements provides quantitative information about the effects of the muscle fiber lattice on actin–myosin binding affinity.

In the earlier phase of this work, conventional EPR spectra of MSL-fibers showed that AMPPNP changes the orientational distribution of heads as inferred from conventional EPR spectra of MSL-fibers (Thomas and Cooke, 1980). The interpretation of that result depends on further information supplied by the present study, in which we have more quantitatively analyzed these spectra, measured saturation transfer EPR on the same samples, and correlated these data with measurement of S1 binding and stiffness. One possibility suggested from the previous work was that the heterogeneous orientational distribution was due to the subsaturating 4 mM AMPPNP concentration. However, in the present study we have used 16 mM AMPPNP and have shown that 84 ± 6% of the actin-bound heads have bound nucleotide, and we have shown that the two orientational populations are present in equal proportions (Fig. 1). One possible interpretation is that AMPPNP simply shifts the equilibrium towards cross-bridge detachment, with all the attached cross-bridges having the same orientation as in rigor. However, this would require that 50% of the cross-bridges are detached under the conditions of Fig. 1, which is not likely in light of the observed rigorlike stiffness. The other possibility, strongly supported by the results of the present study, is the formation of a new cross-bridge conformation in which the myosin head orientational distribution and rotational mobility are different from those in rigor.

The digital analysis of conventional EPR spectra in the presence of 16 mM AMPPNP shows that half of the heads have probes oriented precisely as in rigor (Fig. 2), so this component provides no evidence for a myosin head orientation differing from rigor by more than a few degrees. The remaining disoriented population of heads could be attached but over a wide range of angles. However, V_2' spectra (Fig. 1, *right*) show that the rotational mobility of these disoriented heads is much greater than observed in rigor and is the same as in relaxation. Because the same linear combination (0.475 relaxed and 0.525 rigor) gave an excellent fit for both conventional and ST-EPR spectra, we conclude that half of the heads are dynamically disoriented and are probably detached from actin, undergoing Brownian rotational motion as freely as in relaxation. The conclusion that the disordered heads are detached is more directly supported by the simultaneous measurement of binding and orientation of MSL-labeled S1 in oriented fibers (Figs. 4 and 5). The concentration of disoriented S1 was the same as that of the reservoir in rigor, relaxation, and AMPPNP, whether the temperature was 4 or 18°C, showing that the disoriented heads are not attached to actin. The remaining S1, the highly oriented population (Fig. 5), is oriented precisely as in rigor, despite the near-saturating concentration of AMPPNP, showing that bound heads in the ternary complex indeed have the same orientation as in rigor.

The poor fit of the binding of intrinsic heads to a single population (Fig. 6), coupled with the lack of a stiffness change after almost half the heads were dissociated by AMPPNP, strongly suggests that there are at least two affinities of AMPPNP-bound heads for actin. Our determination of K_3 for S1 · AMPPNP in the fiber (Fig. 4) along with the measurement of intrinsic head binding under the same conditions (Fig. 6) allows the determination of the effective actin concentration seen by the intrinsic heads in a fiber. The effective actin concentration is defined as the concentration of actin that would give the same degree of binding if the affinity were the same for extrinsic and intrinsic heads: A^{eff} = [intrinsic heads attached]/[intrinsic heads detached]/K_3(S1). Alternatively, this can be expressed as different values of K_3: K_3(intrinsic) = [intrinsic heads attached]/[intrinsic heads detached]/[A^{act}] = $K_3(\mathrm{S1})*A^{\mathrm{eff}}/A^{\mathrm{act}}$, where A^{act} is the actual concentration of free actin.

Thus the analysis of Fig. 6 suggests that there are at least two very different values of A^{eff}: at least 5 mM (probably at least 10 mM) for the strongly bound heads and 0.055 ± 0.015 mM for weakly bound heads. This should be compared to an actual total concentration of 0.64 mM in intact fibers (Tregear and Squire, 1973), although the value of A^{act} is probably lower due to swelling of the lattice in glycerinated fibers (Godt and Maughan, 1977), and due to the occupation of some of the actin by attached heads. Our measurement of 240 μM heads (based on the ADPSL titration) combined with [A^{tot}]/[M] = 6.5 (Yates and Greaser, 1983), yields a value of A^{tot} = 780 μM. Correcting for the occupied actin sites, this becomes A^{act} = 680 μM at 4 mM AMPPNP and 660 μM at 16 mM AMPPNP. Therefore, the two populations of heads have K_3 (intrinsic) values of 400 and >40,000 M^{-1} at 4 mM AMPPNP and values of 70 and 7,000 at 16 mM AMPPNP. The two head populations might correspond to two heads of a double-headed cross-bridge. The stronger binding of the first head might be a result of its favorable steric position in the filament lattice, and the weaker binding of the second head could be due to the strain imposed by the mismatch of the actin and myosin filaments when the first head is bound.

Relationship to Other Work

The effects of AMPPNP have been investigated by a variety of techniques in the hope of inducing a long-lived state that corresponds to a key intermediate in the cross-bridge cycle. In the presence of this ATP analogue, a muscle fiber loses about half of its rigor tension, without a corresponding loss of stiffness, an event termed "pseudorelaxation" (Kuhn, 1973, Barrington-Leigh et al., 1972; Marston et al., 1976; Padron and Huxley, 1984). Other characteristic effects of AMPPNP include a small increase in sarcomere length (Marston et al., 1976), the appearance of relaxed-like features in x-ray diffraction (Marston et al., 1976), and the suggestion (from electron micrographs) of a new cross-bridge conformation differing from both rigor and relaxation (Reedy et al., 1965, 1983). From this evidence, it has been proposed that AMPPNP changes the conformation of the attached myosin head from its rigor orientation, at an angle of ~45° with respect to the filament axis, to an angle of ~90°. Such a transition has been proposed to represent a reversal of the power stroke (Marston et al., 1976).

Our results confirm that AMPPNP changes cross-bridge conformation without affecting stiffness, but we find that half of the heads are detached from actin, and the probes on heads remaining attached have precisely the same orientation as in rigor, ruling out a significant axial rotation of the entire head. Although the mismatch of the thin and thick filament periodicities makes cross-bridge heterogeneity likely (Wray, 1984), it is unlikely that half of the cross-bridges are detached, because this would require either that half of the cross-bridges in rigor bear negligible stiffness or that AMPPNP increases the stiffness of those cross-bridges that remain attached to actin. We favor an alternative explanation: AMPPNP induces a single head of each cross-bridge to detach from actin without affecting the stiffness of the cross-bridge. This would imply either that one head of each cross-bridge binds more tightly to actin and bears most of the rigor stiffness, and/or that the compliance of the cross-bridge is in S2, not S1 (Konrad and Goody, 1982). Single-headed cross-bridge binding is consistent with previous studies of the binding of single- and double-headed myosin subfragments to actin. For both heads binding independently and identically K_3^{HMM} should be equal to $(K_3^{\mathrm{S1}})^2$, but K_3^{HMM} has been observed to be much less than $(K_3^{\mathrm{S1}})^2$ (Highsmith, 1978; Greene, 1981; Marston, 1982). It has been reported that ADP can induce rotational motion within MSL-labeled heavy meromyosin (HMM, having two heads) without dissociating HMM from actin (Manuck et al., 1986). Similar effects of AMPPNP were not observed, probably because of (*a*) the weak binding of heads to actin in the presence of AMPPNP at the low actin concentrations possible in solution, and (*b*) the lack of the effects of the filament lattice, in contrast to the present study.

Our finding of partial detachment of myosin heads by AMPPNP is consistent with x-ray and EM data. Recent electron microscopy coordinated with x-ray diffraction of insect flight muscle revealed no evidence for a new conformation of heads in AMPPNP but rather a mixture of rigor and relaxed conformations (Reedy et al., 1983). Low angle x-ray data of rabbit psoas muscle showed a transfer of mass away from the actin filaments, as indicated by decreased intensities of the 11 equatorial and 5.9-nm meridional reflections, and disappearance of the actin-based 7-nm layer line (Padron and Huxley, 1984). Meanwhile, myosin-based (10 equatorial and 7.2-nm meridional) reflections both become more intense. All these changes are consistent with detachment of a fraction of heads. Moreover, the correlation between the mechanical effects and changes in x-ray diffraction patterns is not straightforward because pseudorelaxation is consistently observed in different preparations while the structural changes are observed only in a few cases (Wray, 1984; Padron and Huxley, 1984). For instance, pseudorelaxation is observed for both rabbit psoas and frog sartorius muscles, but the latter does not exhibit such pronounced changes in x-ray diffraction patterns (Padron and Huxley, 1984). At room temperature, insect flight muscle and rabbit psoas have the same diffraction pattern in AMPPNP as in rigor. It is at low temperatures (4°C) that the differences are apparent, although pseudorelaxation occurs irrespective of temperature (Goody et al., 1975; Lymn, 1975). It is not clear that the structural changes as observed in x-ray patterns are necessary for pseudorelaxation.

Our estimate of $K_4 = 330 \pm 130\ \mathrm{M}^{-1}$ (the association constant for AMPPNP binding to the actin-bound myosin head) is in good agreement with the 500 M^{-1} value reported for the dissociation of the A–S1 complex and inhibition of its ATPase activity (Biosca et al., 1986). The inhibition of tension and maximum contraction velocity by AMPPNP also indicate weak nucleotide binding with $K_4 =$ 1,000 and 200 M^{-1}, respectively (Pate and Cooke, 1985). The weak binding is further supported by measurements of the cross-bridge detachment rate, which did not saturate in the millimolar range of AMPPNP (Schoenberg and Eisenberg, 1985). These values are, however, ~100 times higher than those obtained from the binding of radioactive AMPPNP (Marston et al., 1976) or the binding of a fluorescent derivative of AMPPNP (Yanagida, 1981). These reports of high-affinity binding were obtained at submillimolar nucleotide concentrations where the binding curves showed no saturation. More recently, Konrad and Goody (1982) observed that equilibrium binding to A–S1 was 10 times stronger than that determined from kinetic measurements, $k_{\mathrm{off}}/k_{\mathrm{on}} = 1.5$ mM. To minimize chemical heterogeneity, we have used nearly saturating concentration of AMPPNP.

The strength of myosin head binding to actin, as estimated from our MSL-S1 binding experiments, varied between $0.9 \times 10^3\ \mathrm{M}^{-1}$ and $5 \times 10^3\ \mathrm{M}^{-1}$ at 16 mM and 4 mM AMPPNP, respectively. Similar values of K_3 were found for the binding of S1 to regulated actin in the weak form (Greene, 1982). The fivefold difference in binding at the two ligand concentrations probably reflects the activation of actin filaments at lower [AMPPNP], where at least 35% of actin monomers are binding myosin heads or extrinsic S1, as opposed to only 14% at 16 mM AMPPNP. The same difference in actin occupancy resulted in a 2.4-fold change in binding strength of S1 to regulated actin in solution (Greene and Eisenberg, 1980).

The present study is complementary with two other EPR studies on MSL-labeled myosin heads, one on the effects of AMPPNP and $\mathrm{PP_i}$ in myofibrils (Ishiwata et al., 1986), and one on effects of $\mathrm{PP_i}$ in fibers (Pate and Cooke, 1988). In the myofibril study it was shown that AMPPNP induces microsecond mobility of MSL-S1 (in solution with actin), as measured by ST-EPR, only when the S1 is dissociated from actin. In the present study, we have extended this conclusion to S1 in muscle fibers. In the study of $\mathrm{PP_i}$ in fibers, only conventional EPR was measured, but much more extensive stiffness studies were performed (Pate and Cooke, 1988). Due to the low solubility of $\mathrm{MgPP_i}$ it was not possible to approach saturation of the myosin active sites, as was achieved in the present study with AMPPNP.

Glycerol was used by Pate and Cooke (1988) to weaken the actomyosin bond and thereby dissociate most of the myosin heads in fibers, in contrast to the maximal 50% dissociation we obtained in aqueous solution. Despite these and other differences in methodology, the conclusions of Pate and Cooke (1988) are quite consistent with ours: PP_i affects the spectrum of heads only by dissociating them from actin, stiffness is unaffected despite the dissociation of about half the intrinsic heads in fibers; and at least two populations of heads (weakly and strongly binding) must be postulated to fit the data. Whereas neither AMPPNP nor PP_i produces an actin-attached myosin head different from rigor in orientation or motion, as detected by MSL, other EPR studies using MSL have provided evidence that ATP produces an attached head with microsecond rotational mobility, both in muscle fibers at low ionic strength (Fajer et al., 1985) and in cross-linked acto-S1 (Svensson et al., 1986). Thus it appears that the predominant actin-attached states present during the steady-state of acto-myosin ATP hydrolysis are distinct from those produced by AMPPNP and PP_i (Thomas et al., 1988).

Conclusion

In summary, we conclude that in the presence of saturating levels of AMPPNP, half of the myosin heads are detached from actin and have freedom of microsecond rotational motion similar to that of myosin heads in relaxation. The remaining heads are indistinguishable from rigor both in orientation and in mobility. Thus AMPPNP does not cause the entire myosin head to assume a different axial angle of actin attachment from that in rigor. Of course, because our measurements apply directly only to the SH1 region of the head, it is possible (and even likely) that AMPPNP induces significant structural changes in other parts of the head (Cooke, 1986; Thomas, 1987). AMPPNP does induce an attached cross-bridge conformation distinguishable from rigor, namely a single-headed cross-bridge. Although these results apply strictly only in the presence of AMPPNP, they are quite consistent with results in the presence of PP_i (Pate and Cooke, 1987), and they probably indicate that at least two populations of heads, differing greatly in actin affinity and contribution to stiffness, occur under other conditions. A single-headed cross-bridge might be an intermediate in the contractile cycle, helping to explain the apparent discrepancy between high stiffness (Yamamoto and Herzig, 1978) and the lower fraction of oriented heads as observed by EPR (Cooke et al., 1982) in isometrically contracting fibers. Further studies combining structural and dynamic data with measurements of cross-bridge binding and stiffness will be essential in determining the role of myosin's conformational transitions in muscle contraction.

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