Paramagnetic probes attached to a light chain on the myosin head are highly disordered in active muscle fibers

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ABSTRACT We have measured the orientation of a region of the myosin head, close to the junction with the rod, during active force generation. Paramagnetic probes were attached specifically to a reactive cysteine (Cys 125) of purified myosin light chain 2 (LC2) and exchanged into myosin heads in glycerinated rabbit psoas muscle. Electron paramagnetic resonance spectroscopy was used to monitor the orientation of the probes. Previous work has shown that the LC2 bound spin probes are significantly ordered in rigor and muscle in the presence of adenosine diphosphate (ADP). In contrast, there is a nearly random angular distribution in relaxed muscle. We show here that during the generation of isometric tension, all of the LC2 bound spin probes (98 ± 1.6%) show an angular distribution similar to that of relaxed muscle. These findings contrast with results obtained from probes attached to Cys 707 on the cross-bridge, located close to the actin binding site, where, during active force generation, a proportion of the spin probes were ordered as in rigor, whereas the remaining probes were disordered as in relaxation. To test the hypothesis that this ordered component is due to modification of Cys 707, we measured the spectra obtained from probes attached to LC2 in fibers modified at Cys 707. The modification of Cys 707 did not produce an ordered component in these spectra. The absence of an ordered component at the LC2 site limits the populations of some states in active fibers. An actin/myosin/ADP state is thought to be the major force-producing state. Our present results show that the populations of states with ordered probes on LC2 are <2% in active fibers; thus, the major force-producing state is different from the one obtained by addition of ADP to rigor fibers.

INTRODUCTION

Force generation in active muscle is thought to involve a change in orientation of the myosin heads that form cross-bridges between thick and thin filaments (Reedy et al., 1965; Huxley, 1969; dos Remedios et al., 1972). The orientation of myosin heads in muscle fibers has been investigated using a variety of techniques, including electron microscopy, x-ray diffraction, and spectroscopic probes (reviewed by Cooke, 1986). Despite this effort, the exact changes in myosin orientation or conformation that result in force generation remain unknown. Two locations on the myosin head, a reactive sulfhydryl (cys 709) and the nucleotide binding site, have been studied extensively using paramagnetic and fluorescent probes (Borejdo et al., 1979; Yanagida, 1981; Cooke et al., 1982; Burghardt et al., 1983; Crowder and Cooke, 1987; Fajer et al., 1990*a, b*). Both of these sites are located within 5 nm of the region that binds to actin (Sutoh et al., 1984, 1986). All of these studies have agreed that these two sites are relatively highly ordered in rigor or in rigor plus adenosine diphosphate (ADP) and that they are highly disordered in relaxed fibers. In active fibers there is general agreement that there are two populations of probes: one oriented as in rigor or as in rigor plus ADP and one disordered as in relaxed fibers (Yanagida, 1981; Cooke et al., 1982; Burghardt et al., 1983; Fajer et al., 1990*b*). The population of the ordered fraction has been more controversial, with estimates ranging from 12% (Fajer et al., 1990*b*) to >50% (Burghardt et al., 1983). The oriented population of probes must arise from myosin heads attached to actin, and it was thought that the disordered probes arose from myosin heads that were detached from actin. However, the latter conclusion has been questioned by the recent finding that myosin heads can possess microsecond mobility while attached to actin (Svensson and Thomas, 1986; Berger et al., 1989). These data suggest that in active fibers myosin heads can attach to actin in at least two states: one that is very flexible and one in which the distal end (at least) is rigidly oriented on actin. In the present study, we probe the orientation of the proximal end of the myosin head, using a site that is located adjacent to the head-rod junction (Katoh and Lowey, 1989). Previous work has characterized the spectra of rigor and relaxed fibers (Hambly et al., 1991).

EXPERIMENTAL METHODS

Sample preparation

Purified myosin light chain 2 (LC2) was labeled with fluorodinitroanaline spin label (FDNA-SL) as described previously (Hambly et al., 1991). Chemical cleavage at unmodified cysteine, using the cyanalation reaction described by Katoh and Lowey (1989), has established that the FDNA-SL is bound selectively to Cys 125 of LC2. FDNA-SL LC2 was exchanged into glycerinated rabbit psoas muscle fibers using methods developed by Hoffman et al. (1990). Under the conditions used, we estimate from the signal intensity of the ordered component that ~10–15% of fiber LC2 had been exchanged specifically into myosin heads. Mechanical measurements were made on these SL-LC2 fibers as described by Pate and Cooke (1989). Mechanical measurements have shown that the procedures used to exchange LC2 into fibers do not impair fiber function (Hambly et al., 1991); activated tensions were 92% of control values (0.12 mN/mm^2) and relaxed tensions were <0.005 mN/mm^2. These results are in agreement with previous work by Hoffman et al. (1990).

Some SL-LC2 fibers were also modified with chemical probes at the myosin SH-1 site, using the method described by Crowder and Cooke

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(1984), before spectroscopic measurements. Four different protocols were used. One set of fibers was labeled with maleimide spin label (MSL) (*N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl] maleimide) for 30 min, the nitroxide spin label was reduced with ascorbic acid, and the fibers were then exchanged with SL-LC2. The second set of fibers were labeled with MSL that had been reduced with ascorbic acid before labeling and the fibers were then exchanged with SL-LC2. The third and fourth sets of fibers were labeled with either *N*-ethyl maleimide (NEM) for 30 min or iodoacetamide (IAA) for 4 h, after which the fibers were exchanged with SL-LC2. Labeling with either MSL, NEM, or IAA under these conditions has been shown to produce in excess of 80% modification of the Cys 707 residue of myosin (Crowder and Cooke, 1984). Thus, each labeled LC2 would have a probability of 80% of exchanging into a head that was modified, assuming that modification of Cys 707 does not affect LC2 exchange. Since levels of LC2 exchange were similar in modified and in unmodified fibers, it appears unlikely that modification of Cys 707 affects LC2 exchange.

Electron paramagnetic resonance (EPR) spectroscopy

SL-LC2 EPR spectra were obtained at 25°C with a spectrometer (model ER/200D; IBM Instruments Inc., Danbury, CT) interfaced to a PC-AT computer. Each spectrum shows the derivative of absorption plotted as a function of the magnetic field with a sweep width of 9 mT. Two experimental configurations were used. In one protocol, muscle fibers (50–100) were placed in a capillary (0.7 mm ID) that extended through side wall holes (3 mm diam) of a TM110 cavity such that the fibers were aligned parallel to the static magnetic field. The fibers were secured at their ends with surgical silk, and the capillary was attached to a flow system that allowed a rapid perfusion of the fibers. Alternatively, EPR measurements were performed with a TE102 cavity using a specially designed flat cell made of rexolyte. The flat cell allowed the ends of the muscle fibers (7 mm long) to be tied in the cell and incorporated a flow system (using two fine capillaries embedded in the shaft of the cell) to allow rapid perfusion of the fibers while mounted in the cavity of the spectrometer. Fibers in rigor were bathed in "rigor buffer" (0.12 M KCl, 5 mM $MgCl_2$, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 10 mM Na-PO_4, and 20 mM TES, pH 7.0). Relaxed spectra were obtained by the addition of 4 mM adenosine triphosphate (ATP), 0.4 mg/ml^{-1} creatine kinase, and 30 mM creatine phosphate to the rigor buffer. Contraction was induced by adding 1 mM $CaCl_2$ to give a concentration of free Ca^{2+} of $\sim 10^{-5}$ M, sufficient to fully activate the fibers. Full activation of fibers was limited to ~2 min to avoid the possible appearance of large structural sarcomeric inhomogeneities that may occur in fiber bundles contracting at 22°C. After contraction, the fibers were returned to rigor buffer, and data were used only when the reappearance of a well-ordered spectrum indicated that sarcomere heterogeneity had not developed during contraction. Difference spectra were obtained by subtraction of two spectra obtained sequentially from the same fiber under different conditions. The spectra were not manipulated (realigned or intensities adjusted) before subtraction.

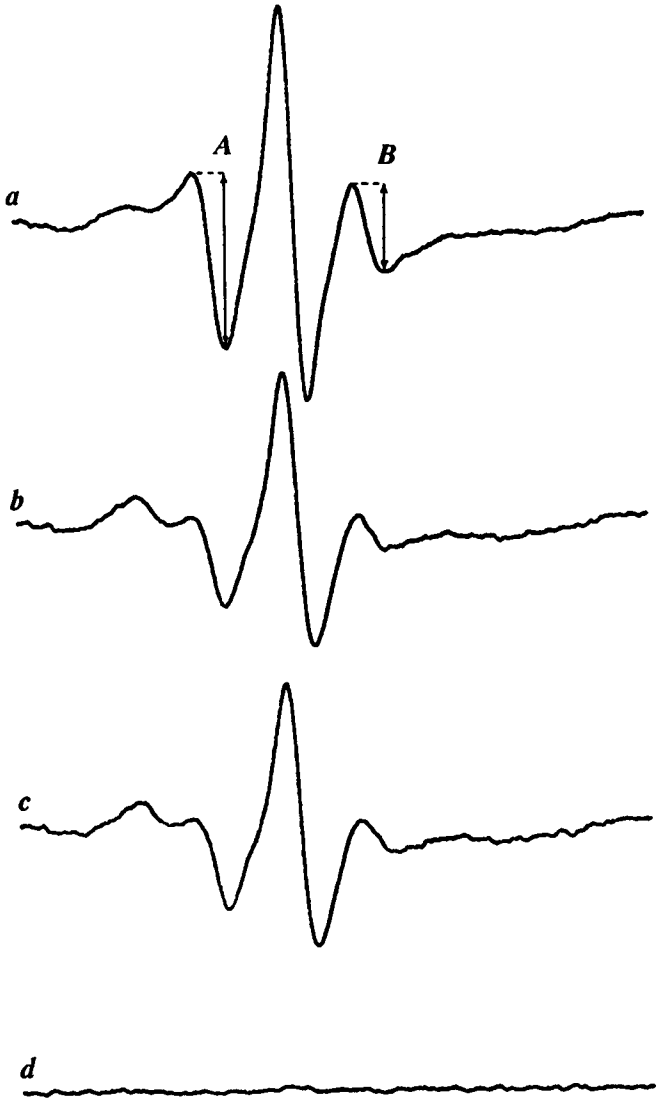

FIGURE 1 EPR spectra of spin labels attached to LC2 of myosin heads during rigor (*a*), relaxation (*b*), and isometric contraction (*c*). The derivative of absorption is plotted as a function of the magnetic field. The width of the spectrum is 9 mT. Spectrum *d* shows the difference spectrum between active and relaxed fibers. Arrows *A* and *B* define the spectral parameters used to quantitate the presence of any ordered rigor peaks in spectra obtained from relaxed and activated fibers.

RESULTS

EPR spectra of FDNA-SL LC2 fibers during active force generation

A FDNA-SL can be reacted with high specificity with one of the two reactive sulphydryls on LC2 (Cys 125), and the labeled LC2 can be exchanged into glycerinated rabbit psoas muscle fibers (Hambly et al., 1991). We report here the use of this LC2-bound spin probe to investigate cross-bridge orientation during isometric contraction of skeletal muscle. Nitroxide spin probes are an excellent tool for measuring protein orientation because their orientation can be determined from their spectra with high resolution (1–2°) (Barnett et al., 1986). When the probes are oriented at one angle, the spectrum consists of three lines whose splitting depends on the angle between the principle axis of the probes and the magnetic field of the spectrometer. When the probes are disordered, the spectrum consists of broad peaks at high and low field.

Fig. 1 *a* shows the EPR spectra of FDNA-SL LC2-labeled muscle fibers held isometrically and parallel to the magnetic field in conditions of rigor. The spectrum of fibers in rigor contains two spectral components. One

TABLE 1 **Ordered component observed in EPR spectra of actively contracting fibers containing exchanged spin labeled LC2**

Spectral parameter	Average ordered component ± standard error
	%
A	3.0 ± 2.1
B	0.9 ± 2.4
Average of both parameters	1.9 ± 1.6
Average of both parameters obtained from fibers also modified with maleimide at the Cys-707 site	0.6 ± 2.9

The ordered component in each active spectrum was determined using the formula: (active − relaxed)/(rigor − relaxed). Measurements were made of the two spectral parameters defined in Fig. 1 and the values averaged ± standard errors. Spectra were obtained from a total of fourteen contractions in the case of fibers that had not been modified at the Cys-707 site and from a total of five contractions in the case of fibers modified with reduced maleimide spin label or *N*-ethyl maleimide at the Cys-707 site.

component, consisting of the three sharp lines in the center of the spectrum shown in Fig. 1 *a*, arises from probes that are oriented. The second component consists of the low amplitude broad peaks seen at high and low field along with some contribution to the sharp center peak. Our previous work has shown that the first component arises from probes on myosin cross-bridges, whereas the second component is most probably due to light chains that are nonspecifically bound to other sites (Hambly et al., 1991). A comparison between relaxed and rigor fibers shows that a given peak height of the ordered fraction in the rigor spectrum represents one-half the number of spins as does the same peak height of the disordered fraction seen in relaxed fibers (where peak height is measured from the baseline to the top of the positive intensity). Using this ratio, we estimated the relative fractions of ordered and disordered components in the rigor spectrum. The spectrum shown in Fig. 1 has 57% in the ordered fraction. In the spectra considered, this varied from 45 to 66% (Fig. 2 shows a rigor spectrum with 66% specific labeling).

Also seen in Fig. 1 are spectra for relaxation (*b*) and tension (*c*). Fig. 1 *d* shows the difference spectrum between relaxed (*b*) and active (*c*) fibers. When ATP is added to relax the fibers, the three ordered sharp lines broaden as shown in Fig. 1 *b*, and the EPR spectrum becomes independent of the angle between the fiber and the magnetic field, showing that the angular distribution of the probes is highly disordered. Addition of Ca^{2+} to relaxed fibers induces tension (Fig. 1 *c*). This spectrum is essentially identical to that obtained in relaxation. The difference between the two spectra was quantitated by measuring spectral parameters at two points (*A* and *B*) defined in Fig. 1 *a*. The two points were chosen to have a maximum sensitivity to the presence of a rigor spectral component. As shown in Table 1, the average of the data obtained at these two points show that at most the rigor component appearing in the spectra of active fibers amounts to 1.9 ± 1.6%. In addition, the difference spectrum between active and relaxed fibers is flat within experimental error as shown in Fig. 1 *d*. Thus, we conclude that no new probe angles, different from those seen in relaxation, are observed in active fibers.

The resolution of the spectrum is high. If >5% of the probes assumed a rigor angular distribution or a new, equally well-defined angular distribution differing from the rigor angular distribution by >10° or if >10% of the probes were less disordered than in relaxation (that is distributed over <90°) during activation, then they would have been detected. To provide a more quantitative estimate, we averaged the difference spectra of six different experiments. These difference spectra were aligned by comparison of their respective rigor spectra. The resulting averaged spectrum is contrasted with a rigor spectrum in Fig. 2. The averaged spectrum is also shown multiplied by 10 to facilitate comparison. It should be noted that any population of ordered probes would give rise to a set of three peaks with approximately equal spacing between them. No such set is seen in the difference spectrum.

Validation of the method used to quantitate the rigor component in EPR spectra

The method we have used here to quantitating the rigor component in spectra obtained from SL-LC2 fibers differs from that used in previous studies at the Cys 709 site on the cross-bridge (Cooke et al., 1982). We chose

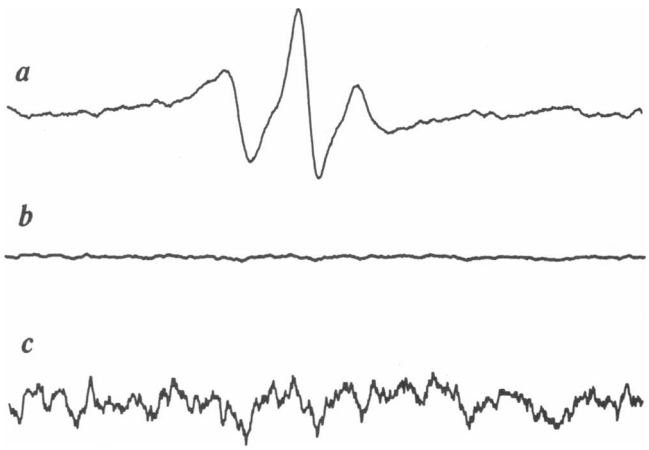

FIGURE 2 A spectrum of fibers in rigor (*a*) is contrasted with an average difference spectrum obtained by subtracting relaxed from active spectra in six different experiments (*b*). The average difference spectrum is multiplied by 10 and shown in *c*. No three-line spectrum characteristic of an ordered population is seen in the difference spectrum.

the present method because it gave the greatest reproducibility in the presence of a variable baseline that occurred in the spectra obtained at high spectrometer gain settings. By measuring the difference between adjacent positive and negative deflections of *A* and *B* in Fig. 1 *a*, baseline fluctuations are minimized. We have determined that a similar measurement between the positive and negative intensities associated with the low field peak of the ordered component in the spectrum gives quantitatively similar results to the previously used method in a series of nine spectra from actively contracting fibers labeled at the SH-1 site. The rigor component in contraction was found to be equal to 22.1 ± 1.9% using the old method versus 22.9 ± 2.8% using our new method.

Analysis of the origin of probe disorder in relaxed and active fibers

The disorder seen in both active and relaxed fibers could be due to disorder in the orientation of a large proportion of the protein structure in this region of the myosin head or it could be due to a more discrete nucleotide-induced change in the conformation of the protein at this site that directly affects mobility of the probe relative to the protein. As discussed in detail in our previous paper (Hambly et al., 1991), the probe has mobility in the nanosecond time-range through relatively large angles (~ ±35°). The narrow lines seen in the rigor spectrum are a result of spin narrowing, i.e., the rapid motion within their rather large cone produces narrow lines with positions that approximate those expected for spin probes oriented at the center of the cone. We tested whether this mobility was altered by the presence or absence of bound nucleotides. The effect of increased probe mobility is to decrease the splitting between the high and low field peaks shown in Fig. 1. We measured the splitting in diced rigor fibers and in relaxed fibers and found that within experimental error (= ±0.05 mT), the splitting between these peaks and the shape of the peaks were unchanged. In addition, we have also measured spectra for labeled myosin heads in solution and again found that within experimental accuracy, the shape of the spectrum remains unchanged by the presence of nucleotides at the adenosine triphosphatase (ATPase) site on the myosin head. We conclude that the presence of a nucleotide does not alter the interaction between the spin probe and the protein surface nor does it alter the mobility in the submicrosecond time scale of the protein sensed by the probe in this region. Because the probe shows an ordered spectrum in the rigor case, it indicates that the relatively high degree of order seen in the rigor spectrum is lost in the spectrum of both relaxed and active fibers due to a disordering of the protein structure in the vicinity of the probe on LC2. This disorder does not involve changes in the interaction between the probe and the protein.

Effect of modification of Cys 707 on EPR spectra during active force generation

The lack of a rigor-like component in the active fibers contrasts with the results obtained using paramagnetic probes attached to the Cys 707 residue, where a fraction of the probes, 10–20%, assume an ordered conformation in active fibers, both with (Cooke et al., 1982) and without (Fajer et al., 1990*b*) ferricyanide treatment to reduce unwanted background labeling. It is known that the presence of a probe at Cys 707 alters the ability of the myosin to function (Svensson and Thomas, 1986; Titus et al., 1989), raising the possibility that the rigor-like component is induced by the probe itself. To investigate this possibility, we exchanged LC2-bound spin probes into fibers that had first been reacted with NEM, MSL, or with MSL reduced to eliminate its EPR signal. The comparison between spectra obtained from relaxed and active fibers after maleimide modification again showed no significant difference, and the data provided in Table 1 limits the presence of a rigor component in the active fibers to 0.6 ± 2.9%. Thus, the presence of a label attached to Cys 707 has not induced a rigor-like component in active fibers as measured by probes attached to the LC2. We note that the spectra shown in Fig. 1 also help eliminate another potential artifact that could have produced the rigor-like component seen in the earlier work at the Cys 707 probe site. An insufficient supply of ATP at the center of the fiber would produce a rigor-like component, and the lack of such a component in the fibers labeled at LC-2 shows that our ATP regenerating system is adequate to ensure that all parts of the fiber are perfused with substrate.

EPR spectra obtained at low ATP concentrations

When fibers are relaxed at low concentrations of ATP, an ordered rigor-like component appears in the spectrum, as shown in Fig. 3 *b* (fibers relaxed in 20 μM ATP). The magnitude of this component is not changed by the presence (*c*) or absence (*b*) of Ca^{2+} as shown by their difference spectrum (*d*). The ordered component in Fig. 3, *b* or *c*, may be deconvoluted from these spectra and, after proportional amplification (*f*), is essentially identical to the original rigor spectrum (*a*). The disappearance of this ordered component of the spectra as the ATP concentration is raised is shown in a double reciprocal plot in Fig. 4. The intercept on the x axis defines an apparent K_m for the interaction of ATP with the spin probe on the LC2, with values of 13.6 μM for relaxed fibers (*solid line*) and 15.5 μM for active fibers (*short-dashed line*). Both these values are similar in magnitude to that obtained for the ATPase activity of fibers and myofibrils (15–20 μM) (Sleep and Glyn, 1986). The similarity in apparent K_m obtained for relaxed and active

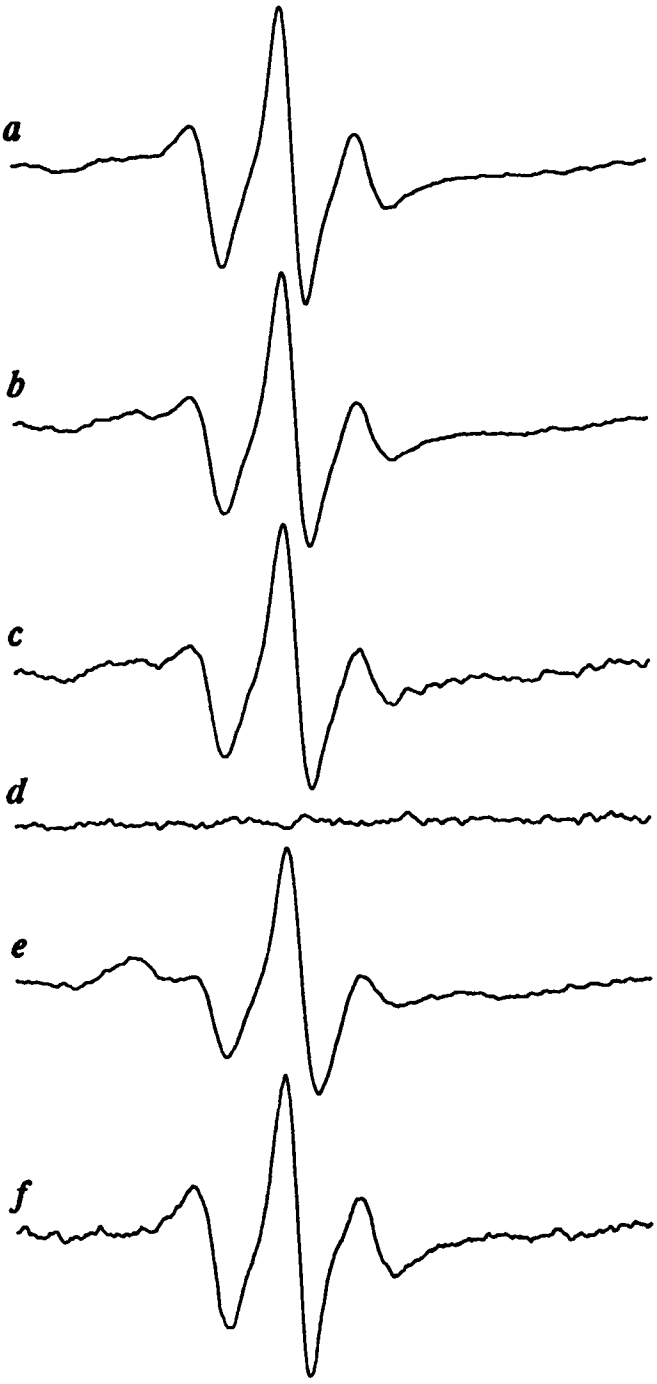

FIGURE 3 EPR spectra are shown of the same preparation of fibers during rigor (*a*) or bathed in 20 μM ATP in the absence (*b*) and presence (*c*) of $\sim 10^{-5}$ M free Ca^{2+}. Spectrum *d* shows the difference spectrum obtained when the relaxed spectrum (*b*) is subtracted from the active spectrum (*c*) obtained in 20 μM ATP. Spectrum *e* shows these same fibers relaxed in 4 mM ATP. Spectrum *f* shows the difference spectrum obtained when the disordered component, 52% of the spectrum obtained at 4 mM ATP (*e*), is subtracted from the spectrum of active fibers obtained at 20 μM ATP. This difference spectrum has been amplified by 2.1 so that the spectral intensities represent the same number of probes as spectra *a–c*. This difference spectrum shows that the ordered component of the spectra shown in *b* (or *c*) is essentially identical to that obtained for rigor fibers shown in *a*.

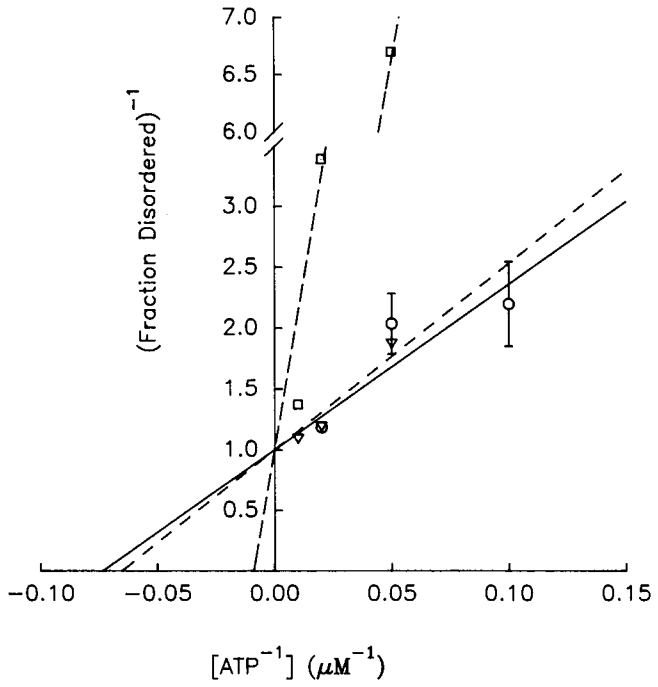

FIGURE 4 The inverse of the fraction of disordered spin probes (±SE) is plotted as a function of the inverse of the ATP concentration over the range 10–100 μM ATP. The fraction of ordered probes was determined by measuring the parameters *a* and *b* shown in Fig. 1 and assuming that the spectrum of rigor fibers represented 100% ordered probes, whereas the spectrum of fibers relaxed in 4 mM ATP represents 0% ordered probes. Conditions are the same as for Fig. 1 *b*, except for ATP concentration. Data were obtained in the absence (○) and presence (▽) of $\sim 10^{-5}$ free Ca^{2+}. Additionally, data derived from fibers in the absence of free Ca^{2+} that had been chemically modified with iodoacetamide (IAA) at the Cys 707 site are shown (□). The straight lines are least-squares fits to the data constrained to pass through one on the abscissa. The intercept on the x axis provides a value of 13.6 mM for K_m in the absence of Ca^{2+} (*solid line*), 15.5 μM in the presence of Ca^{2+} (*short dashes*), and 110 μM in fibers modified with IAA at the Cys 707 site in the absence of Ca^{2+} (*long dashes*).

fibers is expected, since the formation of rigor complexes at low [ATP] (<100 μM) will fully activate the thin filament in a cooperative manner (Murray et al., 1981). Thus, the ordered components seen in Fig. 3 *b* and *c*, most likely represent true rigor heads, that is, heads in the absence of nucleotide, whereas the disordered component represents those heads, either active or relaxed, that have bound nucleotide.

EPR spectra obtained at low ATP concentrations after Cys 707 modification

The apparent K_m observed here of 13–15 μM is smaller than that observed for the interaction of ATP with probes at the Cys 707 site in active fibers (75 μM) (Cooke et al., 1982). The fraction of disordered probes on LC2 in fibers activated at 50 μM ATP (82%) is considerably larger than that obtained for probes at the Cys 707

site (33%) (Cooke et al., 1982). Thus, during activation at this ATP concentration, the majority of the probes at the Cys 707 site are ordered, whereas the majority of probes at the LC2 site are disordered, providing a dramatic difference between the properties of the probes at these two sites in active fibers. The difference in apparent K_m at these two sites may be explained in one of two ways. First, modification of the Cys 707 site with a spin probe may change the actual K_m of acto-myosin for ATP to ~75 μM, or alternatively, at low ATP concentrations there may be an increased population of states in which probes at the Cys 707 site are ordered, whereas probes at the LC2 site are disordered. To test these alternatives, we labeled SL-LC2 fibers with IAA at the Cys 707 site (~80% of Cys 707 is modified under these conditions) (Crowder and Cooke, 1984). Thus, 80% of the labeled LC2 should be on modified myosin heads. As shown in Fig. 4, the double reciprocal plot of ATP concentration versus the fraction of disordered probes yields a K_m for ATP binding, from the SL-LC2 spectra for these fibers, to be ~110 μM (Fig. 4, *long-dashed line*), demonstrating that Cys 707 modification has in fact changed the K_m of acto-myosin for ATP to ~100 μM.

DISCUSSION

Relationship to other probe studies of LC2

Two other groups also have investigated spectroscopic probes attached to myosin light chains in muscle fibers. Arata (1990) attached a MSL to LC2 in a manner similar to that used here. He observed a rather disordered probe distribution in rigor fibers, in contrast to the order observed with the FDNA-SL. He observed some order in the presence of a nonhydrolyzable analogue of ATP, AMPPNP. It is possible that the disagreement between this work and the present work arises because the maleimide probe is on a different cysteine. Arata claims to have labeled Cys 155, whereas the probe used here is attached primarily to Cys 125. In a preliminary report, Shrimpton et al. (1990) have shown that a fluorescent probe attached to LC2 at the Cys 155 is ordered in rigor fibers, disordered in relaxed fibers, and partially ordered in active fibers. The results obtained in rigor and relaxation agree with those found for the FDNA-SL. Resolution of the discrepancy found in active fibers must await further work but again may be due to the probe location; their probe is attached to Cys 155, whereas ours is on Cys 125. It would appear that the results obtained to date may depend on the type and location of the probes employed.

Structural changes during active force generation

Previous spectroscopic probe results at the Cys 707 and ATP sites have suggested that a considerable fraction of the myosin heads (10–50%) can assume an ordered state in active fibers (Yanagida, 1981; Cooke et al., 1982; Burghardt et al., 1983; Thomas, 1987; Fajer et al., 1990*b*). Spin probes attached to Cys 707 have a population with an angular distribution that is close to that seen for rigor cross-bridges. The proportion of cross-bridges in this ordered state during force generation has been found to be between 11 and 25% at 22°C (Cooke et al., 1982; Fajer et al., 1990*b*; Pate, E., and R. Cooke, unpublished data). A fluorescent probe attached to Cys 707 also displays an ordered component in active fibers, although here the probe is sensitive to the presence of ADP, and the ordered component has an orientation similar to that observed in the presence of ADP. The population of the ordered state is >50%, considerably more than that seen with spin probes. Fluorescent nucleotides in active fibers have also displayed an ordered component with an orientation similar to that seen for the diphosphate nucleotide bound to rigor fibers (Yanagida, 1981). At the low concentrations used, the ordered component was large, ~50%.

We have addressed the possibility that the ordered component arising in active spectra is a consequence of the labeling of Cys 707, by first labeling Cys 707 with spectroscopic or chemical probes, before examining the orientation and motion of our spin label attached to LC2. Modification of the Cys 707 residue failed to induce an ordered component in our spectra derived from the LC2 spin probe site at millimolar ATP concentrations in active fibers. Clearly, modification of the Cys 707 residue with extrinsic probe molecules does not artifactually induce a population of cross-bridges that are oriented in the rigor conformation in active fibers, either transiently or permanently. We assume here that a rigor cross-bridge is one in which spin probes attached to either the Cys 707 residue or LC2 are ordered at their respective rigor angles. However, it is possible that the proportion of the population of cross-bridges that assume this rigor-like state during contraction may either be increased or decreased as a result of Cys 707 modification (Svensson and Thomas, 1986; Titus et al., 1989). Consequently, the oriented probes seen at the Cys 707 probe site during contraction report on the orientation of only the part of the cross-bridge that contains the Cys 707 residue, a region that lies proximal to the actin binding site.

We conclude that the probes attached to LC2, near the head-rod junction, are reporting an orientational distribution that is qualitatively different from that observed for probes at the Cys 707 site, close to the actin binding site near the other end of the myosin head. In active muscle, a region of the cross-bridge proximal to the thick filament, including LC2, is disordered during essentially all parts of the contractile cycle, in contrast to a region of the head proximal to the thin filament that attaches to actin in a fixed, well-defined orientation during at least the latter part of the contraction cycle. The population of

ordered probes at Cys 707 found in some studies, however is small, emphasizing an important point that in active fibers the cross-bridges are largely disordered (Fajer et al., 1990*a*).

The results obtained here, along with those obtained previously, suggest that in active fibers, myosin initially forms a weak bond with actin, in which both ends of the cross-bridge that have now been probed are disordered. Subsequently, myosin forms a strong bond with actin, corresponding to the latter part of the attached phase of the contraction cycle. These data suggest that this strong acto-myosin bond corresponds to the distal region of the head being attached strongly to actin in a specific orientation (similar to the rigor or rigor-ADP orientation), whereas the proximal region of the head close to the junction with the rod is disordered. Such a structure would involve considerable distortions of the myosin head in the region between the two ends of the head that have now been probed. This distortion may involve a large region of the myosin head structure or it may be confined to a local region close to the probe site on LC2. Defining the extent of this region of the cross-bridge will require further investigation, although methods such as electron microscopy and x-ray diffraction suggest that the ordered region is small, since these methods predict that most of the mass of the cross-bridge is largely disordered in active fibers (Huxley and Faruqi, 1983; Reedy et al., 1965).

Relationship between structural and biochemical events in active cross-bridges

Studies of the kinetics and energetics of the actomyosin interaction both in solution and in fibers have suggested that force generation involves a transition through a series of states in which the actomyosin bond becomes increasingly stronger (Hibberd et al., 1985; Cooke, 1986). In many models of this interaction, the major force-generating state is one involving an actin-myosin-ADP complex (Eisenberg et al., 1980; Hibberd et al., 1985; Hibberd and Trentham, 1986; Pate and Cooke, 1989). In addition, there is some evidence from studies of fiber mechanics that an actin-myosin-ADP state occurs at the end of the powerstroke. The value of K_i, which describes the effective binding constant for the inhibition of fiber velocity, is equal to the value of K_d measured for the binding of ADP to acto-S1 or to fibers (Pate and Cooke, 1988). This is in agreement with modeling studies suggesting that an actin-myosin-ADP state is a major component of the power stroke (Eisenberg et al., 1980; Hibberd et al., 1985; Hibberd and Trentham, 1986; Pate and Cooke, 1989). The structure of one actin-myosin-ADP state is relatively well characterized. In the state attainable by addition of ADP to rigor fibers, spin probes at the Cys 707 and the LC2 sites are all ordered, showing that the myosin head is attached rigidly to actin in a unique conformation similar to rigor (Fajer et al., 1990*a*; Hambly et al., 1991). Previous probes attached at the Cys 707 and ATP sites had suggested that a considerable fraction of the myosin heads (10–50%) could be in such an ordered state in active fibers (Yanagida, 1981; Cooke et al., 1982; Burghardt et al., 1983; Thomas, 1987; Fajer et al., 1990*b*). However, the present results place an upper limit of ~2% on the total populations of the rigor and the rigor plus ADP states. This leads to the conclusion that the major force-producing states must involve a configuration very different from rigor or from that achieved by addition of ADP to rigor fibers. Studies of the kinetics of the actomyosin interaction have suggested that several actin-myosin-ADP states exist, which cannot be populated by addition to ADP to a rigor complex (Sleep and Smith, 1981; Geeves, 1991). Our data suggest that a major portion of the force is produced in such states and that probes attached to the LC2 are disordered in them.

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