Orientation of spin-labeled light chain-2 exchanged onto myosin cross-bridges in glycerinated muscle fibers

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ABSTRACT Electron paramagnetic resonance (EPR) spectroscopy has been used to study the angular distribution of a spin label attached to rabbit skeletal muscle myosin light chain 2. A cysteine reactive spin label, 3-(5-fluoro-2,4-dinitroanilino)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (FDNA-SL) was bound to purified LC2. The labeled LC2 was exchanged into glycerinated muscle fibers and into myosin and its subfragments. Analysis of the spectra of labeled fibers in rigor showed that the probe was oriented with respect to the fiber axis, but that it was also undergoing restricted rotations. The motion of the probe could be modeled assuming rapid rotational diffusion (rotational correlation time faster than 5 ns) within a "cone" whose full width was 70°. Very different spectra of rigor fibers were obtained with the fiber oriented parallel and perpendicular to the magnetic field, showing that the centroid of each cone had the same orientation for all myosin heads, making an angle of ~74° to the fiber axis. Binding of light chains or labeled myosin subfragment-1 to ion exchange heads immobilized the probes, showing that most of the motion of the probe arose from protein mobility and not from mobility of the probe relative to the protein. Relaxed labeled fibers produced EPR spectra with a highly disordered angular distribution, consistent with myosin heads being detached from the thin filament and undergoing large angular motions. Addition of pyrophosphate, ADP, or an ATP analogue (AMPPNP), in low ionic strength buffer where these ligands do not dissociate cross-bridges from actin, failed to perturb the rigor spectrum. Applying static strains as high as 0.16 N/mm^2 to the labeled rigor fibers also failed to change the orientation of the spin label. Labeled light chain was exchanged into myosin subfragment-1 (S1) and the labeled S1 was diffused into fibers. EPR spectra of these fibers had a component similar to that seen in the spectra of fibers into which labeled LC2 had been exchanged directly. However, the fraction of disordered probes was greater than seen in fibers. In summary, the above data indicate that the region of the myosin head proximal to the thick filament is ordered in rigor, and disordered in relaxation.

INTRODUCTION

It has been postulated that the force-producing event in muscle contraction involves the rotation of a myosin cross-bridge while it is attached to an actin filament (Reedy et al., 1965; Huxley and Simmons, 1971; Morales et al., 1982). To test this hypothesis, cross-bridge orientations have been monitored using electron microscopy, x-ray diffraction, and spectroscopic probes attached to the myosin head. The latter studies have concentrated on the spectra of fluorescent and paramagnetic probes attached to a reactive sulphydryl (SH-1) of the myosin heavy chain and on fluorescent and paramagnetic nucleotides that bind to the ATPase site on the myosin head. Both of these sites are located on the distal region of the myosin head, close to the site where it interacts with actin. We report here that we have bound a paramagnetic probe to isolated rabbit skeletal muscle regulatory light chain 2 (LC2). After exchanging the modified LC2 onto the cross-bridges of glycerinated muscle fibers, this probe has been used to measure the orientation of the proximal portion of the myosin molecule, close to the head-rod junction.

The alkali and regulatory light chains bind to the 20-kDa proteolytic fragment of the myosin heavy chain and together they form the neck region of the cross-bridge (Winkelmann et al., 1983). This region has been called the "regulatory domain" of myosin, because of its functional significance in vertebrate smooth muscle. LC2 is believed to be located near the head-rod junction, based on electron microscopic studies using LC2 depleted myosin subfragments (Vibert and Craig, 1982), and antibodies specific for LC2 (Flicker et al., 1983). Antibodies specific for fluorophores attached to the two Cys residues of skeletal LC2 have also been mapped by electron microscopy close to the head-rod junction and close to each other (Katoh and Lowey, 1987).

The function of LC2 in skeletal muscle fibers remains unclear. Skeletal muscle LC2 binds one mole of divalent cation per mole of LC2 and is reversibly phosphorylated at Ser-19. LC2 depleted fibers show an increase in stiffness and tension compared to control fibers during contraction (Moss et al., 1982) and phosphorylation of LC2 in fibers increases force levels at submaximal Ca^{2+} concentrations (Persechini et al., 1985), suggesting that LC2 may be involved in modulating the calcium sensitivity of cross-bridge cycling in skeletal muscle.

Studies of the orientation of the neck region in rigor

muscle are of particular interest because this region may be distorted by formation of actomyosin bonds. The helical periodicity of the actin and myosin filaments do not match one another, thus, considerable distortion is required to obtain a specific actomyosin interaction (reviewed by Haselgrove and Reedy, 1978; Cooke, 1986). This distortion probably occurs in contractile or elastic elements in the myosin molecule, although some flexibility may exist within the actin polymer (Egelman et al., 1982; Ford et al., 1985). Rigor cross-bridge disorder has been detected by both electron microscopy and x-ray diffraction (Taylor et al., 1984; Haselgrove and Reedy, 1978; Poulson and Lowey, 1983; Heuser and Cooke, 1983). Different orientations for different cross-bridges have been observed in electron micrographs of rigor muscle, with the difference in the orientation being most prominent in that portion that is proximal to the thick filament (Taylor et al., 1984). In contrast, fluorescent and paramagnetic probes at both the reactive sulfhydryl and the nucleotide sites report a high degree of order in rigor fibers (Borejdo and Putnam, 1977; Yanagida, 1981; Thomas and Cooke, 1980; Barnett et al., 1986; Crowder and Cooke, 1987; Haselgrove, 1980; Reedy et al., 1965; Tregear and Mendelson, 1975). Both of these sites are close to the actin site on the myosin head. Together these results lead to the hypothesis that the proximal portion of the myosin head, which is close to the head-rod junction, may be distorted in rigor fibers, a hypothesis that can be tested by placing probes on this region.

Although the structure of relaxed muscle has been studied by a variety of methods, no clear picture of the disposition of the myosin heads has emerged. EPR spectroscopy of spin labels attached to SH-1 in relaxed muscle fibers indicated a high degree of disorder in the cross-bridge (Thomas and Cooke, 1980; Arata and Shimizu, 1981). Saturation transfer EPR (ST-EPR) of the maleimide spin label attached to SH-1 of myosin showed that the spin label is rigidly attached to myosin and that the cross-bridges were undergoing Brownian rotations in a time scale of 1–10 μs (Thomas et al., 1980). Similarly, EPR spectra of fibers relaxed by trapping spin-labeled ADP with vanadate at the myosin ATPase site showed a wide distribution of probe angles (Crowder and Cooke, 1987). Studies of fluorescent probes also indicated much more disorder in relaxed muscle than in rigor muscle fibers (Borejdo and Putnam, 1977; Burghardt et al., 1983; Yanagida, 1981). In contrast, x-ray diffraction and electron micrographs of relaxed muscle have indicated cross-bridge order, in which the myosin heads are arranged in a helical array about the thick filament (Haselgrove, 1980; Cantino and Squire, 1986).

These data, taken together, support the hypothesis that the cross-bridge may be composed of at least two functional regions. The region proximal to actin appears to be highly ordered in rigor fibers, whereas the region of the cross-bridge proximal to the thick filament is expected to be less ordered during rigor to allow for the cross-bridge distortion required by the helical mismatch between the thick and the thin filaments. During relaxation the actin binding region of the cross-bridge is highly disordered, as demonstrated by spectroscopic probes attached to this region. In contrast, data from nonspectroscopic probe sources would predict that some region of the cross-bridge, possibly proximal to the thick filament shows thick-filament-based order.

To directly test these hypotheses, we have attached a spin probe to LC2 and exchanged the spin-labeled LC2 into glycerinated muscle fibers. Because LC2 is located within the region of the cross-bridge proximal to the thick filament, the LC2 bound spin probe can be used to determine the angular distribution of this region in both rigor and relaxed muscle fibers. In contrast to the predictions of the above hypotheses, the probed region of myosin appears to be ordered in rigor fibers and disordered in relaxed fibers.

EXPERIMENTAL METHODS

Striated muscle fibers were derived from rabbit psoas muscles. Small strips of psoas muscle fibers (1–2-mm diam) were dissected, tied to wooden sticks and incubated at 0°C in 50% glycerol/50% solution of 0.24 M potassium acetate, 10 mM magnesium acetate, 10 mM EGTA, 40 mM TES, pH 7. After 24 h, the solution was changed and the muscle fibers were stored at −20°C. The day before use, the fibers were incubated in rigor solution (0.12 M potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, 20 mM TES, pH 7) and 0.02% Triton X-100 at 0°C overnight and then washed with rigor solution before use. Sodium orthovanadate was purchased from Fisher Scientific Co. (Pittsburgh, PA). Stock solutions of 60-mM vanadate were prepared by weight and boiled at pH 10 to destroy polymeric species. AMPPNP (β,γ-imido-ATP) was purchased from Sigma Chemical Co. (St. Louis, MO).

Rabbit skeletal muscle light chain mixture was prepared as described by Holt and Lowey (1975). LC2 was separated from the light-chain mixture using either an analytical cation exchange HPLC column (Biorad Corp., Richmond CA) or by Cibacron Blue affinity chromatography (Toste and Cooke, 1979). LC2 was stored frozen in 50 mM sodium phosphate pH 6.5, 0.5 mM dithiotheitol (DTT) at ~200 μM. Rabbit skeletal muscle myosin was prepared by a modification of the method of Tonomura et al. (1966). Myosin subfragment-1 (S1) was prepared by papain digestion of myofibrils as described by Cooke (1972).

Immediately before labeling, LC2 (~200 μM) was chromatographed through Sephadex G-25M into 50 mM sodium phosphate pH 6.5 and the concentration adjusted to ~150 μM. 0.5 mM 3-(5-fluoro-2,4-dinitroanilino)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (FDNA-SL) (Aldrich Chemical Co., Milwaukee, WI) was added with vortexing at 0°C. The reaction was stopped after 3 h by chromatography through G-25M using LC2 Binding Buffer (10 mM TES pH 7, 25 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EGTA) as an eluent. Typically, ~0.5 mol of FDNA-SL per mole of LC2 was bound. Unlabeled LC2 was separated

from FDNA-SL LC2 on an analytical cation exchange HPLC column. LC2 was applied to the column in buffer A (10 mM sodium phosphate pH 7, 0.25 mM EDTA, and 0.25 mM DTT) and eluted from the column using a linear gradient from 0–50% of buffer B (0.5 M NaCl in buffer A).

FDNA-SL LC2 was exchanged into glycerinated rabbit psoas muscle fibers essentially using the method described by Moss et al. (1982). Bundles of fibers (~ 25–50) were tied to glass rods at rest length and extracted in LC2 fiber extraction buffer (10 mM sodium phosphate pH 7, 50 mM KCl, 20 mM EDTA) at 35°C for 2 h with shaking and with one change of extraction buffer. After extraction, the fibers were washed in LC2 binding buffer for 10 min at 4°C then incubated overnight in LC2 binding buffer containing 50–100 μM FDNA-SL LC2 at 4°C. After exchange, fibers were washed overnight in 50% glycerol/rigor containing 5 mM DDT at 4°C, then stored in 50% glycerol/rigor at −20°C for up to 2 mo before use.

Before exchange of FDNA-SL LC2 into S1 it was found to be essential to separate LC2 depleted S1 from dissociated LC2 using DE-52 chromatography. Failure to perform this step resulted in virtually no exchange of FDNA-SL LC2 into the S1, even in the presence of a large excess of FDNA-SL LC2 during binding to S1. Papain S1 containing FDNA-SL LC2 was prepared by extracting ~ 1 mg/ml S1 in 2 mM EDTA, 2 mM ATP, 40 mM KCl, 10 mM TES, pH 7 for 10 min at 37°C. To separate dissociated LC2 from LC2-depleted S1 the extracted S1 solution was immediately applied to a 2.5 ml DE-52 column in buffer A (50 mM Tris pH 8) at 22°C and eluted within 20 min of extraction using a linear gradient (40 ml each of 0–0.5 M KCl in buffer A). LC2 depleted S1 present in the first protein peak was then mixed with a five times molar excess of FDNA-SL LC2 and 4 mM $MgCl_2$ at 4°C. The FDNA-SL LC2/S1 mixture was exhaustively dialyzed overnight at 4°C against KCl free rigor (20 mM TES pH 7, 5 mM $MgCl_2$, 1 mM EGTA) to remove any residual ATP. FDNA-SL LC2 exchanged S1 was bound to glycerinated muscle fibers overnight at 4°C in KCl free rigor containing 1 mM DTT. Exchange of O-FDNA-SL LC2 into myosin was done by the method of Wikman-Coffelt et al. (1979).

EPR measurements were made with an ER/200D EPR spectrometer from IBM Instruments, Inc., (Danbury, CT). X-Band, first-derivative absorption EPR spectra were obtained with the following instrument settings: microwave power, 25 mW, modulation, 0.2–0.25 mTesla at 100 kHz. EPR spectra from spin-labeled proteins in solution were obtained by mounting a 0.7-mm capillary containing the solution in a TE_{102} cavity. Muscle fiber EPR spectra were obtained in one of three experimental configurations. In one protocol, muscle fibers (50–100) were placed in a capillary (0.7-mm inside diameter) that extended through side wall holes (3-mm diam) of a TM_{110} cavity such that the fibers were aligned parallel to the static magnetic field (H_o). 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 TE_{102} cavity using one of two specially designed flat cells made of rexolyte. In one flat cell, small bundles of fibers (7-mm long) were first blotted, enclosed in the flat cell and oriented parallel or perpendicular to the magnetic field. The second 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. Experiments in which tension was applied to the fiber were done with a force transducer mounted on the cavity. All spectra were obtained at 25° ± 2°C unless otherwise stated. Temperature was maintained by slowly blowing cooled nitrogen gas through the cavity and monitoring by a small thermister embedded in the rexolyte cell adjacent to the fibers.

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

RESULTS

Labeling of LC2

FDNA-SL reacts primarily with Cys risidues but is also reactive with Lys residues. The UV absorption spectrum of FDNA-SL bound to protein varies depending on the residue to which it is bound (Perkins et al., 1984). The absorption spectrum obtained from FDNA-SL LC2 (Fig. 1) was characteristic of the modification of Cys residues. Rabbit skeletal muscle LC2 contains two Cys residues at positions 128 and 157, of which 157 has been shown to be the more reactive with fluorescent probes (Yamamoto et al., 1980). Labeling LC2 at pH 6.5, below the pKa of Cys residues and well below the pKa of Lys residues, increased the specificity of labeling and resulted in almost all of the bound probe being relatively immobile (Fig. 3 *A*). This immobilized component of the spectrum was found to have a rotational correlation time of 8 ns (Goldman et al., 1972), similar to that expected for LC2 free in solution, implying that the spin probe is probably largely immobilized on LC2. The probe mobility was greatly decreased by binding of the light chain to ion exchange beads (Fig. 3 *B*). Probe mobility is discussed in more detail in a later section. Labeling LC2 at pH 8 resulted in the appearance of a second spin probe population, of considerably greater mobility (rotational correlation time < 0.1 ns), indicating that under these conditions at least two residues had been labeled.

To optimize FDNA-SL LC2 exchange into muscle fibers, FDNA-SL LC2 was separated from unlabeled LC2 by cation exchange HPLC (Fig. 2). Although the separation achieved was relatively poor, there was a significant improvement in the subsequent exchange of FDNA-SL LC2 into fibers. The order of elution was: unlabeled LC2 (*Shoulder A*), FDNA-SL LC2 (*Shoulder B*), a mixture of labeled and unlabeled LC2 dimers (*Peak C*) and larger oligomers (*Peak D*). The extent of labeling

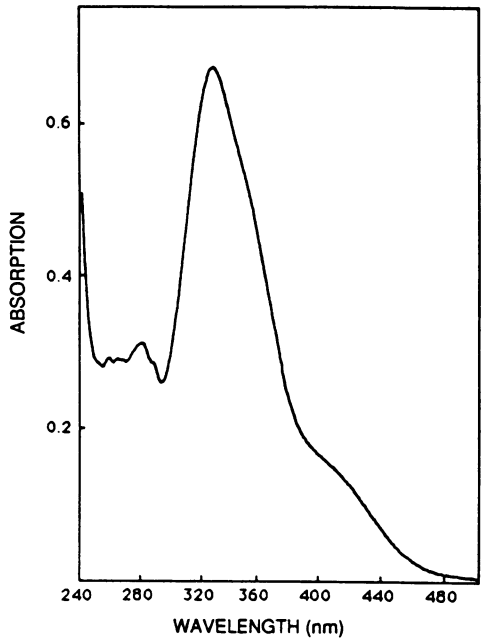

FIGURE 1 Absorption spectrum of 32 μM FDNA-SL LC2, containing 1 mol FDNA-SL bound per mole of LC2, in LC2 binding buffer, 1 cm pathlength. The maximum absorption of the fluoro-dinitroanaline ring at 330 nm is characteristic of binding to a sulfhydryl group rather than an amino group (two peaks at 345 and 425 nm) (Perkins et al., 1984).

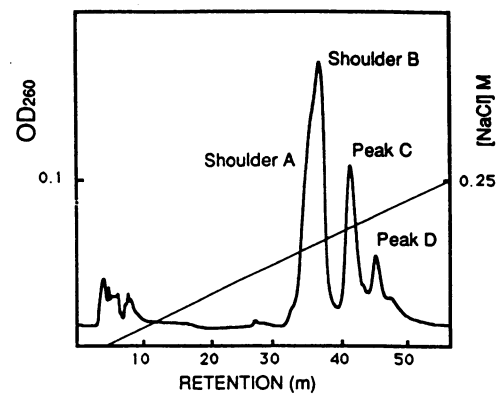

FIGURE 2 Elution profile of the separation of FDNA-SL LC2 from unlabeled LC2 by cation exchange HPLC. The labeled LC2 mixture was applied to the column at time 0 in buffer A (10 mM sodium phosphate, pH 7, 0.25 mM EDTA, and 0.25 mM DTT) and eluted from the column using a linear gradient between times 4 and 54 min of 0–50% buffer B (0.5 M NaCl in buffer A). Shoulder A contained unlabeled LC2, Shoulder B contained FDNA-SL LC2, Peak C contained dimerized LC2 (mixture of labeled and unlabeled LC2), and Peak D contained larger oligomers of LC2. Although the separation between the FDNA-SL LC2 and unlabeled LC2 was poor, there was still a significant improvement in the exchange of FDNA-SL LC2 into muscle fibers after separation.

in Shoulders A and B was determined spectrophotometrially using an extinction coefficient at 330 nm of 21,100 M^{-1}. The extent of oligomerization in Peaks C and D was determined using nonreducing SDS-polyacrylamide gel electrophoresis. To avoid reduction of the paramagnetic nitroxide radical, FDNA-SL LC2 could not be stored in reducing agents, such as DTT, for prolonged periods of time. However, both labeled and unlabeled LC2 readily forms oligomers in the absence of reducing agents (over 24–48 h), necessitating labeling, separation and exchange into fibers being performed without delay. Optimal exchange conditions required the extraction of LC2 from fibers by heating of the fibers to 35°C for 2 h after the methods of Moss et al. (1982). Under the conditions used we estimated from the signal intensity that ~10–15% of the LC2 had been exchanged.

Mechanical effects of LC2 exchange

Mechanical measurements showed that the procedures used to exchange LC2 into fibers did not impair fiber function. Fiber stiffness measurements performed on exchanged fibers showed that the fibers relaxed normally when perfused with relaxing solution (rigor solution containing 4 mM ATP, 0.4 mg/ml creatine kinase, and 5 mM creatine phosphate). Active tension developed by

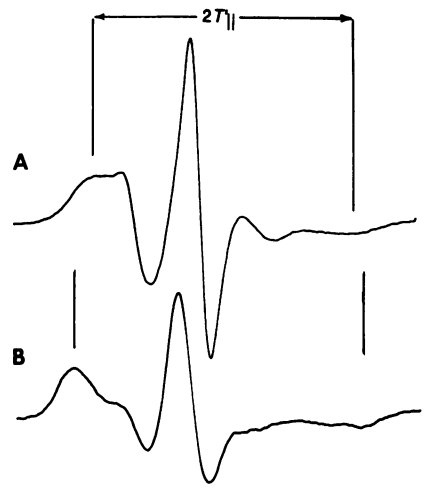

FIGURE 3 EPR spectrum of FDNA-SL LC2 in binding buffer. In this and subsequent spectra, the derivative of the absorption is plotted as a function of the magnetic field. The width of each spectrum is 9.0 mTesla. $2T'_{\|}$ represents the hyperfine splitting, from which the rotational correlation time of the bound probe may be estimated. (*A*) FDNA-SL LC2 displaying a value of $2T'_{\|}$ of 5.9 mTesla corresponding to a rotational correlation time of 8 ns. (*B*) FDNA-SL LC2 bound to DE-52 beads. The value of $2T''_{\|}$ has increased to 6.6 m Tesla indicative of a rotational correlation time of about 30 ns.

the exchanged fibers was between 80 and 100% of control (unexchanged) values, and was not different from fibers that were exchanged with unlabeled LC2. Due to the low level of labeled LC2 in these fibers it is difficult to determine whether labeling has affected the ability of the head to generate force; however, the normal relaxed tensions achieved suggest that the labeled heads did relax properly.

EPR spectrum of FDNA-SL LC2 rigor fibers

Fig. 4 shows EPR spectra obtained from a bundle of rigor fibers into which FDNA-SL LC2 has been exchanged. The fibers are mounted either parallel (Fig. 4 *A*) or perpendicular (Fig. 4 *B*) to the static magnetic field (Ho). Information on the orientation of bound probes is provided by a comparison of the EPR spectra obtained with the magnetic field applied to the muscle fiber at different orientations. The large differences between parallel and perpendicular EPR spectra indicate that the probes are significantly ordered with respect to the magnetic field. The line shape of the EPR spectrum obtained with the fibers mounted parallel to the magnetic field (Fig. 4 *A*) shows two components. One (apparently ordered) population of probes gives rise to the three sharp lines in the center of the spectrum. A second (apparently disordered) population of probes is evident from the broad peaks seen at high and low field (and also contributing some intensity to the central peak). When the labeled fibers are aligned perpendicular to the magnetic field, the EPR spectrum again contains two components (Fig. 4 *B*). The three sharp lines in the parallel spectrum, representing the oriented population of probes, become markedly broadened in the perpendicular spectrum, demonstrating a dramatic dependence upon orientation of the muscle fiber in the magnetic field. However, the disordered population of probes seen in the parallel spectrum is not changed significantly in the perpendicular spectrum, demonstrating that this component is approximately randomly oriented.

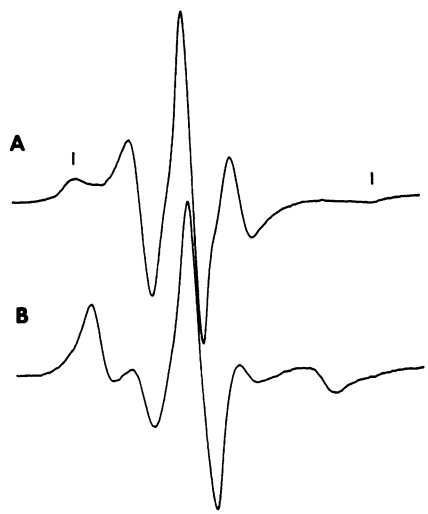

FIGURE 4 EPR spectra of FDNA-SL LC2 exchanged into glycerinated rabbit psoas muscle fibers bathed in rigor buffer. Spectrum A is oriented parallel to the magnetic field ($H_\parallel$) and spectrum B is oriented perpendicular ($H_\perp$). The considerable difference in the line shape of the two spectra demonstrates that the probe is oriented with respect to the magnetic field and hence the fiber axis. The vertical lines indicate the approximately isotropic immobilized component seen in both spectra, that is thought to represent LC2 bound nonspecifically to the fiber.

Angular distribution and motion of the probes

The shape of the EPR spectrum is sensitive to both the motion and the orientation of the spin probes. The rotational correlation time of an isotropic population of spin probes in a sample can be estimated by measuring the decrease in the apparent hyperfine splitting ($2T'_\parallel$); defined in Fig. 3 from the rigid limit value $2T_\parallel$ as described by Goldman et al. (1972) and in the legend to Table 1. The value of $2T_\parallel$ for the probe attached to LC-2 was estimated to be 6.9 m Tesla from the spectrum obtained from LC-2 precipitated in ammonium sulfate, (Table 1). This splitting of 6.9 m Tesla suggests that the spin probe is rigidly immobilized on the ns-μs time scale, and any decrease in $2T'_\parallel$ below this value is taken as evidence for nanosecond rotational motion.

The splitting of the approximately random component observed in fibers (discussed above) was close to the rigid limit, suggesting that these probes are relatively immobile. Although it is often obscured by other peaks, this component appears to be present in all other spectra observed, and as discussed below this component probably does not arise from LC2 bound to myosin. Thus, the discussion below will focus on the more mobile probes that are known to be attached to the cross-bridges.

The EPR spectrum of FDNA-SL LC2 exchanged rigor fibers that were finely diced and randomized yielded a splitting of 5.5 m Tesla, demonstrating that the FDNA-SL is undergoing rapid motion in the submicrosecond time scale. Assuming isotropic motion of the probe the formula of Goldman et al. (1972) would yield a rotational correlation time of 5 ns. Given the large difference between the rigor EPR spectra obtained when the intact fibers were mounted parallel or perpendicular to the magnetic field, the motion of the spin probe must be markedly anisotropic and restricted in amplitude. Simulation of the angular distribution of a

TABLE 1 FDNA-SL LC2 Spectral Parameters

	$2T'_{\parallel}$ m Tesla	τ_c s
FDNA-SL LC2	5.91 ± 0.2	8×10^{-9}
FDNA-SL LC2 + Sat. Ammonium Sulfate	6.9 ± 0.1	—
FDNA-SL LC2 + 10% PEG	6.5 ± 0.2	25×10^{-9}
FDNA-SL LC2 + DE-52 beads	6.6 ± 0.1	30×10^{-9}
FDNA-SL LC2 + CM-cellulose beads	6.7 ± 0.1	50×10^{-9}
FDNA-SL LC2 Myosin	5.6 ± 0.1	6×10^{-9}
FDNA-SL LC2 Myosin + Sat. Amm. Sulfate	6.7 ± 0.1	50×10^{-9}
FDNA-SL LC2 S1	5.6 ± 0.3	6×10^{-9}
FDNA-SL LC2 S1 + DE-52 beads	6.7 ± 0.1	50×10^{-9}
Random Diced FDNA-SL LC2 Rigor Fibers	5.5 ± 0.1	5×10^{-9}
Relaxed FDNA-SL LC2 Fibers	5.5 ± 0.1	5×10^{-9}

Spectral parameters for the EPR spectra of FDNA-SL LC2 are listed under a variety of conditions. The splitting between hyperfine extrema $2T'_{\parallel}$ was measured from conventional V1 spectra as shown in Fig. 3. Rotational correlation times τ_c were calculated from values of $2T'_{\parallel}$ using $\tau_c = a(1-2T'_{\parallel}/2T_{\parallel})^b$, where $a = 5.4 \times 10^{-10}$ s and $b = -1.36$ (Goldman et al., 1972). A value of 6.9 m Tesla was used as the rigid limit for $2T_{\parallel}$, determined from FDNA-SL LC2 that had been precipitated in saturated ammonium sulfate. Because the motion of the probe is restricted in amplitude, these vales of τ_c represent an upper limit on the actual value. The errors given for $2T'_{\parallel}$ represent the standard error of the mean for at least five measurements.

population of spin probes diffusing rapidly through a variety of constrained angles can be ambiguous. The spectra expected for probes diffusing rapidly within a cone has been calculated by Griffith and Jost (1976). Two spectral parameters 2 $A_{\parallel}$ (the splitting between the two outer peaks) and 2 $A_{\perp}$ (the splitting between two inner peaks) were measured and compared to their rigid limit values (determined in saturated ammonium sulfate). Assuming rapid diffusion within a cone and using Eq. 38 from Griffith and Jost, the full width of the cone through which the probes are diffusing can be estimated. These calculations suggest that the spin probe is diffusing rapidly within a cone whose full width is ~70°.

Simulations of the spectra expected for oriented spin probes in rigor fibers have been undertaken previously by Thomas and Cooke (1980) and Barnett et al. (1986). A prerequisite for these particular simulations is that the spin probes are rigidly immobilized on the microsecond time scale. In an ordered immobilized system, the angle between the center of the orientational distribution of the principal axis of the spin label and the static magnetic field (θ) can be determined by measuring the splitting between the high and the low field peaks, and the width of the Gaussian distribution ($\Delta\theta$) can be most easily determined by comparing the peak heights of the low and high field peaks to that of the central peak. However, if the spin probe in an ordered system is not rigidly immobilized on the microsecond time scale, but is undergoing restricted, submicrosecond anistropic motion, the line shape of the EPR spectrum is markedly changed. For rapid restricted motion, the major change in the spectral line shape, as the frequency of motion increases, is a sharpening of all three ordered lines of the EPR spectrum. The angle between the magnetic field and the centroid around which the spin probe is rapidly diffusing is difficult to calculate accurately. If it is approximated by assuming that the splitting between the three sharp lines represents the average value of θ (θ_{ave}) experienced by the probe; the value for θ_{ave} is 74°. Whereas the absolute value of θ_{ave} is uncertain, the narrow width of the three sharp lines in Fig. 4 *A*, along with the ratios of the peak heights, requires that the θ_{ave} of each head is approximately the same.

Analysis of the origin of probe motion in rigor fibers

The rotational correlation time of the FDNA-SL at the LC2 site in rigor fibers is considerably faster than has been observed for probes bound to the SH-1 and ATP sites of rigor fibers. It is therefore important to establish whether the probe motion observed in the EPR spectra of rigor fibers is reporting directly on the motion of the region of the head to which the probe is attached, or whether the probe itself is making a significant contribution to the observed motion. One approach to differentiating between these possibilities is to measure probe mobility under conditions that would be expected to selectively reduce the rotation of the protein itself without changing any local rotation of the probe. The rotational correlation time of FDNA-SL LC2 free in solution (8 ns) is close to that expected for a globular protein of this molecular weight, suggesting that motion of the probe itself is making little or no contribution to the observed rotational correlation time.

Two methods were used to selectively reduce protein rotation (Table 1). FDNA-SL LC2 and FDNA-SL LC2 exchanged into S1 were bound to ion exchange beads (Fig. 3 *B*) or precipitated with ammonium sulfate or polyethylene glycol (PEG). The probes bound to each of the isolated proteins in solution all displayed rotational correlation times in the submicrosecond range (high to low field splitting < 6 m Tesla). However, upon immobilizing each labeled protein on either anion or cation exchange beads, there was a substantial reduction in probe mobility in all cases. Precipitation of the various

labeled proteins with ammonium sulfate resulted in a larger reduction in probe mobility approaching the value of the rigid limit. The reduction in mobility could be modeled as a change in the rate of motion or as a change in the amplitude of the motion. In Table 1 the mobility changes are characterized in terms of τ_c, the rate of motion, assuming isotropic (unrestricted) rotation. In all cases, protein immobilization results in correlation times that are much longer than observed in fibers. It would seem unlikely that the spin probe immobilization observed in all cases tested above would be due to a direct interaction between the spin probe and the beads or a conformational change at the spin probe binding site that specifically restricted the motion of the spin probe. In particular, precipitation with PEG should not affect probe mobility relative to the protein. In summary, much of the motion observed in myosin or in fibers is probably due to motion of the region of the head that has been labeled. This motion could arise in part due to motion of the light chain relative to the heavy chain. The rapidity of the motion would suggest that the portion of protein that is moving is very flexible.

Origin of the disordered immobilized component of the rigor spectrum

The rigor EPR spectrum shown in Fig. 4 includes a population of probes that are disordered and highly immobilized (displaying little mobility in the submicrosecond time range). The exact location of these probes in the fiber is uncertain, although at least three subpopulations of probes may exist: (*a*) probes that are bound to other residues of LC2 (e.g., the second Cys residue or Lys residues); (*b*) probes that are bound to LC2 that has nonspecifically associated with either residual fiber membranes or other fiber proteins; and (*c*) probes bound to cross-bridges that have assumed an alternative physiological conformation, presumably in exchange with the population that gives rise to the anisotropic component of the spectrum.

Factors found to be important in reducing the proportion of disordered probes in the rigor spectrum (up to 80%) include (*a*) separating unlabeled from labeled LC2 before exchange, and (*b*) washing labeled fibers after exchange with 50% glycerol/rigor containing 5 mM DTT overnight at 4°C, followed by further washing in 50% glycerol/rigor for several days or up to 2 mo at −20°C. The effects of these two factors imply that the affinity of labeled LC2 for the myosin HC is lower than that of unlabeled LC2, and suggest that a significant proportion of the disordered component of the rigor EPR spectrum is due to aggregation of the labeled LC2 and/or nonspecific association of the labeled LC2 with various components of the fiber. Given the marked variability in the ratio of the disordered to the ordered components of the rigor EPR spectrum, depending on the methods used to prepare the fibers, we conclude that the disordered component is probably due to nonspecific binding of LC-2 to components in the fiber and does not represent an alternative rigor conformation of the cross-bridge. In support of this conclusion, extracting myosin by incubation in rigor buffer plus 0.5 M KCl and 2 mM PPi eliminated the ordered component of the spectrum while leaving most of the disordered component.

EPR spectrum of FDNA-SL LC2 exchanged S1 bound to fibers in rigor

The line shape of the EPR spectra of FDNA-SL LC2 exchanged S1 bound to fibers in rigor was qualitatively similar to the line shape of FDNA-SL LC2 exchanged rigor fibers, both when the fibers were oriented parallel and perpendicular to the magnetic field (see Fig. 5). The main difference was the presence of a larger population of disordered probes in the labeled S1. However, the ordered component seen in rigor fibers can also be seen in the fibers loaded with S1. Addition of MgPPi in rigor buffer washed out both the ordered and disordered components, showing that both arose from S1 bound to actin. The disordered component may arise from improper binding of LC2 to S1 or to denatured S1. Alternatively, the LC2 region of myosin may be more

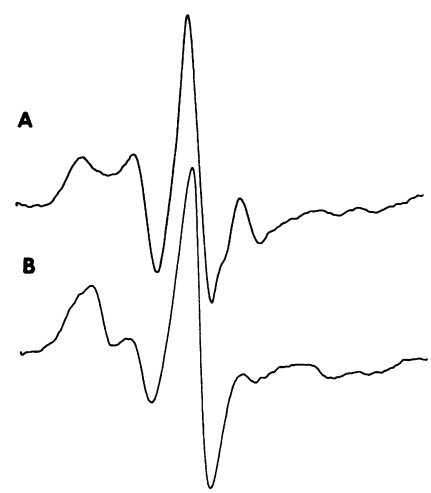

FIGURE 5 EPR spectra of FDNA-SL LC2 exchanged into S1 which was subsequently bound to glycerinated muscle fibers bathed in rigor buffer. In spectrum A, the fibers were oriented parallel to the magnetic field; spectrum B perpendicular to the magnetic field. The sharp lines characteristic of the ordered component of rigor fibers can be seen, along with an enhanced disordered component.

disordered when the myosin head is not connected to a thick filament.

Effects of ATP and ATP analogues

The addition of relaxing solution containing ATP produces a spectrum (Fig. 6, *C* and *D*) whose spectral components closely resemble the spectrum obtained from randomly distributed probes in diced randomized rigor fibers (Fig. 6 *B*). Spectra taken with the magnetic field parallel and perpendicular to the fiber were very similar (compare Fig. 6, *C* and *D*), suggesting that the population of probes are largely disordered in the relaxed state. The small differences seen between parallel and perpendicular spectra can be attributed to a small population (~ 10%) of probes that are oriented as in rigor). These spectra are consistent with the heads detaching from the actin filaments and undergoing large angular motions.

An alternative to ATP induced relaxation is the use of ADP-vanadate to relax fibers. After nucleotide hydrolysis, vanadate forms a long lived complex with myosin-ADP, which inhibits further ATPase activity (Goodno and Taylor, 1982). In insect flight muscle, ADP-vanadate inhibits contraction, and the muscle stiffness and x-ray diffraction patterns mimic the relaxed state (Goody et al., 1980). Relaxation of fibers by binding vanadate-ADP to myosin produced the same spectra as that obtained in ATP relaxing solution.

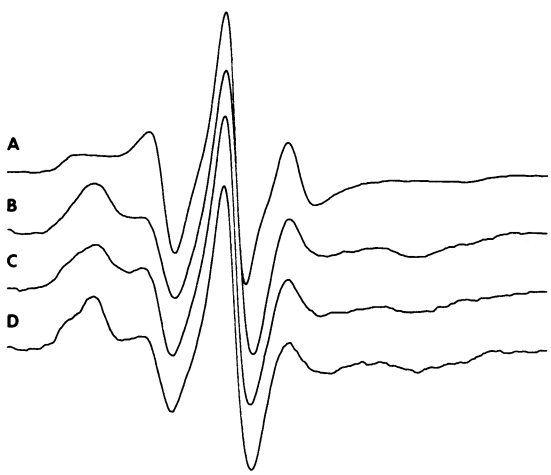

FIGURE 6 EPR spectra of FDNA-SL LC2 bound to glycerinated muscle fibers. Spectrum A was obtained when the fibers were bathed in rigor buffer and mounted parallel to the magnetic field. Spectrum B was obtained when fibers bathed in rigor buffer were diced and randomized before mounting in the EPR cell. Spectra were also obtained in relaxing buffer (rigor buffer plus 4 mM ATP, 0.4 mg/ml creatine kinase, and 5 mM creatine phosphate) with the fibers mounted parallel to the magnetic field, spectrum C; or perpendicular to the magnetic field, spectrum D. The spectra obtained for relaxed and diced randomized rigor fibers are close to isotropic, consistent with the cross-bridges of the relaxed fiber being detached from the thin filaments and undergoing large angular motions.

Nucleotide analogues have been used to produce cross-bridge conformations that may correspond to important intermediates in the cross-birdge cycle. A number of structural studies have indicated that the cross-bridge undergoes a change in conformation in response to the binding of either of the two nucleotide analogues $MgPP_i$ and nonhydrolyzable MgAMPPNP. Under low ionic strength conditions no cross-bridges dissociate from actin in the presence of either of these analogues (Pate and Cooke, 1988; Fajer et al., 1988). The EPR spectra of FDNA-SL LC2 exchanged fibers in the presence of either 16 mM AMPPNP or 4 mM PP_i in low ionic strength rigor buffer (20 mM TES pH 7, 5 mM $MgCl_2$, 1 mM EGTA at 25°C) were indistinguishable from rigor EPR spectra, demonstrating that the FDNA-SL LC2 spin-probe site is also unable to detect a nucleotide analogue induced conformational change in the cross-bridge. Similarly, the addition to fibers of 1 mM ADP plus the myokinase inhibitor AP_5A (0.1 mM) in low ionic strength rigor buffer failed to alter the EPR rigor spectrum, in agreement with stiffness and x-ray diffraction data (Rodger and Tregear, 1974; Marston et al., 1976). Raising the ionic strength of the rigor buffer containing AMPPNP resulted in a decrease in the oriented probe population and a corresponding increase in the disordered population, consistent with heads dissociating from actin.

Effect of stress

Active force is thought to be generated by a change in cross-bridge conformation, leading to the possibility that a passive force could in turn alter cross-bridge conformation. We investigated the influence of external tension on the region of the cross-bridge which binds FDNA-SL LC2 by monitoring the EPR spectrum of this probe as a function of stress applied to the muscle fiber. Muscle fibers in rigor buffer were mounted in a capillary that extended through the microwave cavity such that the long axis of both the capillary and the muscle fibers were parallel with the magnetic field. One end of the bundle of fibers (~ 75 fibers) was secured with surgical thread to a force transducer. The other end was tied with surgical thread that was passed over a pulley and attached to a weight. The EPR spectrum was monitored as stress was applied to the fiber. The spectrum was recorded with no stress on the fiber and again while stress was being applied. With minimal stress, a slight sharpening of lines was detected with no change in the splitting between the three central peaks of the rigor spectrum. We believe

the sharpening of the lines is due to better alignment of the fibers with respect to the magnetic field (Cooke, 1981). Tension was increased in small increments to ~80% of active tension (0.16 N/mm^2), and no additional changes were detected in the EPR spectrum. A comparison of the experimental spectra with a series of simulated spectra leads to the conclusion that a change of $>2°$ in the angle of the axis of the cone of probe motion would have generated a detectable change in the signal. We conclude that a passive tension, which was close to that generated by active fibers (0.2 N/mm^2), does not appreciably alter the angular distribution of the FDNA-SL attached to the myosin cross-bridges.

Proximity of the FDNA-SL to the Ca^{2+} binding site of LC2

LC2 has a site for binding divalent cations which has high affinity for Ca^{2+}, Mg^{2+}, or Mn^{2+} (Minowa et al., 1983). According to the theory of Leigh and co-workers (Leigh, 1970), quenching of the spectral amplitude of the rigor fiber spectrum would be expected to occur if Mn^{2+}, bound to this site, is close to the spin label. No quenching was observed, when 0.1 mM Mn^{2+} in 0.1 M KCl 60 mM TES was added to labeled rigor fibers, indicating that the FDNA-SL is >2.5 nm from the divalent metal binding site of LC2 in agreement with previous conclusions (Minowa et al., 1983). Further addition of 1.0 mM ADP and 0.1 mM AP_5A also did not quench the spin-label spectrum showing that the probe is also not close to the MnADP complex at the ATPase site on the myosin.

DISCUSSION

LC2 binds to the 20-kDa fragment of the cross-bridge which is located in the region proximal to the myosin thick filament (Winkelmann et al., 1983). The spin probe is probably located quite close to the head-rod junction, because both of the two Cys residues of LC2 have been located within 3 nm of the head-rod junction (Katoh and Lowey, 1987). The LC2 spin probe is therefore located a large distance from the previously probed SH-1 and ATP binding sites, essentially on the other end of the myosin head.

The spin probe appears to be preferentially bound to one Cys residue, probably Cys-157, although it is possible that a small proportion of the probe is bound to Cys-128. EPR spectra are capable of separately resolving individual populations of ordered spin probes, hence, the anisotropic component seen in the rigor EPR spectrum and presumed to be derived from probes selectively bound to Cys-157 of LC2 can be separately resolved and monitored under varying physiological states. There is also a disordered component in the rigor EPR spectrum, which is thought to arise from labeled LC2 nonspecifically associated with residual fiber membranes or other fiber proteins. Whereas considerable efforts were made to eliminate the isotropic component of our rigor EPR spectrum, we were only partially successful in achieving this.

In rigor fibers, the LC2 bound FDNA spin probe appears to be diffusing rapidly within a "cone" with a full width of 70°, around a centroid oriented at ~74° to the fiber axis. The "cone" in which the spin probe is diffusing may be circular, elliptical or may have a more complex shape. The motion of the probe within the "cone" has a rotational correlation time shorter than 5 ns, which differs from the much slower rotational correlation time (longer than 1 ms) observed for spectroscopic probes bound to the SH-1 site of rigor cross-bridges. The difference observed by spectroscopic probes located in these two regions of the cross-bridge in rigor fibers may represent a real difference in the mobility of these two regions of the cross-bridge. Alternatively, the faster mobility of the FDNA-SL LC2 spin probe may result from rotation of the probe relative to the protein, or motion of the labeled part of LC2 relative to the cross-bridge. To discriminate between these possibilities, probe mobility was measured under conditions that would be expected to selectively affect protein mobility. In all cases, including spin labeled S1, probe mobility was reduced significantly, suggesting that the FDNA-SL bound to LC2 in rigor fibers is not undergoing motion independent of the region of the cross-bridge to which the probe is attached. Therefore, the simplest interpretation of these data is that in rigor fibers at least part of the region of the cross-bridge proximal to the thick filament and containing LC2 is diffusing rapidly around a well-ordered centroid within a "cone" of ~70°. It is possible that this region of LC-2 is mobile relative to the heavy chain of myosin.

The cross-bridges of rigor fibers are most probably found in several configurations, these configurations being the product of two forces, the constraints of the filament lattice and the energetics of the strong bond between actin and myosin. Electron microscopic evidence suggests that heterogeneity in rigor cross-bridge orientation is most prominent in the region proximal to the thick filament (Taylor et al., 1984) of the cross-bridge. The LC2 based spin probe is bound to this region of the cross-bridge and therefore in rigor may be expected to display some heterogeneity in probe orientation. However, although this portion of the protein is mobile, the highly oriented spectral component shows

no evidence for more than one narrow distribution in rigor.

Although some structural techniques, x-ray diffraction, and electron microscopy, suggest that myosin heads are ordered about the thick filament in relaxed muscle, spectroscopic probe studies have failed to discriminate any ordered fraction of relaxed cross-bridges (reviewed by Cooke, 1986). Because the FDNA-SL LC2 spin probe is located in a different region of the cross-bridge to those spectroscopic probes previously studied, it seemed likely that an ordered fraction of the LC2 based spin probes would be observed in relaxed fibers. However, the EPR spectrum of FDNA-SL LC2 spin probes in relaxed fibers was largely indistinguishable from diced randomized rigor fibers, indicating that the FDNA-SL LC2 probe population in relaxed fibers is highly disordered, at both physiological and low ionic strengths.

There are many possible explanations for the absence of an ordered fraction in the relaxed state. The two heads of the ordered fraction of relaxed cross-bridges resolved by x-ray diffraction and electron microscopy may fold back on the filament axis at different angles to the fiber axis. If there is a sufficient heterogeneity in the angles of each of these two head populations, then an apparently disordered distribution of probes may result. Another population of probes may be weakly bound to the thin filament, but either at a number of different orientations or with considerable motional freedom, hence, appearing to be disordered. Finally, a population of cross-bridges may not be associated with either the thick or the thin filaments and hence will be disordered.

The binding of the nucleotide analogues adenylyl-5′-imidodiphosphate (MgAMPPNP) or pyrophosphate ($MgPP_i$) to the myosin cross-bridge produces quaternary complexes with binding strengths intermediate between rigor and relaxation (Biosca et al., 1986). Structural studies using intact fibers, x-ray diffraction, and electron microscopy have clearly shown that MgAMPPNP produces changes in cross-bridge structure upon binding (Goody et al., 1976; Lymm, 1975; Marston et al., 1976; Padron and Huxley, 1984; Reedy et al., 1983). However, EPR spectroscopy of fibers labeled at the SH-1 site has failed to detect any conformational state other than rigor amongst the population of cross-bridges that remain bound to actin in the presence of saturating concentrations of nucleotide analogues (Pate and Cooke, 1988; Fajer et al., 1988). One possible explanation for these data is that the structural changes observed by other methods may occur in a region of the cross-bridge that does not contain the SH-1 site and that this region is likely to be proximal to the thick filament. The FDNA-SL LC2 based spin probe also failed to detect any change in the orientation or motion of this region of the head when nucleotide analogues bound to fiber heads, suggesting that if a nucleotide analogue induced structural change occurs in the head it does not involve the region adjacent to the head-rod junction.

The application of stress to fibers failed to perturb the rigor EPR spectrum of the FDNA-SL LC2 probe. Similar results have been found using fluorescent and spin probes bound to both the myosin nucleotide site and the SH-1 residue and for fiber tryptophans (Yanagida, 1983; Cooke, 1981; Crowder and Cooke, 1987; dos Remedios et al., 1972). Measurements of the transient mechanical response of active fibers after step changes in muscle length have shown that some compliance exists within the head (Huxley and Simmons, 1971; Ford et al., 1985). An apparently similar compliance is also found in rigor fibers (Tawada and Kimura, 1984). The results obtained with probes at all three head sites (SH-1, ATP and LC2 sites) would suggest that the observed compliance is not located in the vicinity of any of these probe sites. Because the SH-1 and nucleotide binding sites are located at least 5 nm from various sites of actin (Takashi, 1979; Trayer and Trayer, 1983; dos Remedios and Cooke, 1984) and the LC2 probe appears to be at or close to the head-rod junction (Katoh and Lowey, 1987), it is difficult to imagine that the compliance observed physiologically is located within the head itself; this compliance is probably located in some other region of the myosin molecule, possibly in the S2 region. This hypothesis is consistent with small-angle x-ray diffraction data which failed to observe any radial redistribution of cross-bridge mass when stress was applied to fibers (Naylor and Podolsky, 1981).

Our aim in labeling fiber cross-bridges with an LC2 based spin probe was to examine the orientation and motion of the region of the head proximal to the thick filament, the so called "regulatory domain" (Winkelmann et al., 1983). Whether the FDNA-SL LC2 we have exchanged onto fiber heads is actually reporting on the majority of this region is not certain. However, the protein immobilization experiments we performed to clarify this question strongly suggest that the probe does report directly on the motion of the region of protein to which it is attached. Assuming that this conclusion is correct, the simplest model of the head that emerges from these data suggests that the head consists of at least two regions, a region proximal to actin and a region proximal to the thick filament. In rigor the region proximal to actin is rigidly immobilized. On the other hand, the region proximal to the thick filament that was probed in this study, appears in rigor to be capable of considerable motion and flexibility within the constraints of a "cone" of $\sim 70°$. Despite the apparent flexibility of this region in rigor cross-bridges, the application of strain to the fiber was unable to alter the orientational distribution of the probes. Either the

distortion is confined to a small fraction of the myosin heads, or the compliance arises from the S-2 region of the myosin. During relaxation the probe population in the region we have probed proximal to the thick filament assumes a disordered distribution and hence appears to be capable of motion through large angles. Finally, this region of the head does not appear to assume any new conformational state when nucleotide or nucleotide analogues are bound to heads that are bound to actin, suggesting that the conformational change associated with force generation may be primarily located within some other region of the head. Clearly, further probe studies at this and other sites on the light chains of the myosin cross-bridge will help to clarify the function of this important region of the cross-bridge.

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