Orientation of Paramagnetic Probes Attached to Gizzard Regulatory Light Chain Bound to Myosin Heads in Rabbit Skeletal Muscle†

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ABSTRACT: The orientation of the myosin neck was monitored using electron paramagnetic resonance (EPR) spectroscopy. Gizzard regulatory light chain was labeled with a nitroxide spin probe and exchanged for the native subunit, located in the myosin neck, in rabbit psoas muscle fibers. The EPR spectra of rigor fibers indicated a substantial degree of probe immobilization and showed a strong dependence on the orientation of the fiber axis relative to the magnetic field, indicating that the neck was ordered in this state. Spectra of relaxed fibers at 24 °C showed that the neck was disordered, but the spectra of relaxed fibers at 4 °C indicated that the neck was partially ordered. Active fibers at the two temperatures produced spectra identical to relaxed fibers, indicating that no novel angles could be seen in the neck during the powerstroke. Proteolytic fragments of myosin, S1 and HMM, were exchanged with labeled light chains and bound to thin filaments in unlabeled fibers. The distribution of probe orientations for HMM was identical to that of labeled rigor fibers, while S1 showed a slightly different distribution, suggesting that the neck is distorted (by a few degrees) by the interactions of the two heads of myosin when bound to actin.

Force and motion generation by contracting muscle is thought to involve a change in orientation of myosin heads that form cross-bridges between thick (myosin) and thin (actin) filaments [reviewed by Cooke (1986) and Goldman (1987)]. Recently, three-dimensional structures have been determined for both actin and the myosin head (Holmes et al., 1990; Kabsch et al., 1990; Rayment et al., 1993b). The skeletal muscle myosin head (S1)[1] is composed of two domains: a large, globular region which binds both actin and nucleotides, the catalytic domain, and a slender neck region, which connects the catalytic region to the rod region. The myosin neck consists of a portion of the myosin heavy chain, formed by a single α helix, and two light chains; the essential light chains and the regulatory light chains (RLC). The helix extends from the catalytic domain for a distance of 85 Å and ends at the junction with the rod, which leads to the thick filament. The two light chains wrap around this helix to provide bulk and stability to the neck region (Rayment et al., 1993b).

The determination of the three-dimensional structures of the contractile proteins led to an atomic level model for force generation (Rayment et al., 1993a). This model has been refined in light of subsequent structures of the myosin catalytic domain bound to nucleotides. The model suggests that conformational changes in the catalytic domain, mediated by hydrolysis and release of the γ-phosphate of MgATP, result in a swing of the myosin neck, producing a 5−10 nm powerstroke. Data from spectroscopic probes [reviewed by Cooke (1986)] and from recent structural studies (Rayment et al., 1993) have suggested that the catalytic domain of myosin remains rigidly attached to actin during force generation. Therefore, the model predicts a considerable change in the orientation of the myosin neck during the powerstroke.

Two structural studies suggest that the orientation of the neck can change. Fluorescent probes attached to RLC were found to rotate transiently upon step changes in muscle length, an indication that RLC changes orientation during the powerstroke (Irving et al., 1995). However the actual observed change in orientation was small (3−5°), complicating the interpretation. The necks of smooth muscle and a non-muscle S1 attached to actin were observed to change orientation upon addition of ADP (Jontes et al., 1995; Whittaker et al., 1995). Studies of myosin function have also suggested that the neck acts as a lever arm. When the length of the neck is changed by extracting light chains or by adding or deleting light chain sites on the heavy chain, the velocity generated in an *in vitro* translation assay correlated well with the length of the neck (Itakura et al., 1993; Lowey & Trybus, 1994; Uyeda, 1996).

Electron paramagnetic resonance (EPR) spectroscopy provides a powerful tool for investigating the orientation and mobility of specific sites on macromolecules such as proteins. Using EPR, changes in orientation within 3−5° and rotational motion with correlation times in the range from 10^{-12} to 10^{-6} s can be determined (Barnett et al., 1986; Thomas, 1987). Previous EPR studies on muscle have mainly concentrated on the myosin catalytic domain (Cooke et al., 1982; Fajer et al., 1990b; Thomas & Cooke, 1980; Zhao et al., 1995). Probes attached to Cys-707, located in the catalytic region of myosin, were highly oriented in rigor and

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[1] Abbreviations: EPR, electron paramagnetic resonance; IASL, 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy (spin label); HMM, heavy meromyosin; RLC, myosin regulatory light chain; S1, myosin subfragment 1.

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disordered in relaxation (in the presence of ATP), indicating that the myosin catalytic domain is ordered in rigor and undergoes random Brownian motion in relaxation. In active fibers (in the presence of ATP and Ca^{2+}), about 12%−20% of the probes were ordered with the same orientation as seen in rigor, and the remaining probes were disordered, similar to those in relaxation (Cooke et al., 1982; Fajer et al., 1990b; Roopnarine & Thomas, 1995; Zhao et al., 1995). Selective population of a pre-powerstroke state by addition of high P_i indicated that this state was also oriented as in rigor, confirming that the orientation of the catalytic domain did not change during the powerstroke (Zhao et al., 1995). Together these data led to the conclusion that the catalytic domain remained rigidly oriented on actin for at least part of the powerstroke, in support of the model presented by Rayment et al. (1993a). Both the earlier probe data and the more recent protein structures have led to the conclusion that the myosin neck forms the lever arm envisioned by the rowing oar model of cross-bridge function.

The orientation of the neck has been investigated using a variety of structural techniques, including EPR spectroscopy. Rabbit skeletal light chain 2 (RLC) was extracted, labeled with a paramagnetic probe on Cys-125, and exchanged back into fibers (Hambly et al., 1991). In the absence of nucleotide the EPR spectra of the probe depend strongly on the orientation of the fibers axis relative to the magnetic field (parallel *versus* perpendicular), indicating that RLC and thus the neck are ordered in rigor. The probe is highly disordered in relaxed fibers, in agreement with observations of the catalytic domain. In contrast to the results found for the catalytic domain, no ordered fraction is found in active fibers, suggesting that the neck is highly disordered during force generation (Hambly et al., 1992). This conclusion is compatible with the hypothesis that the neck acts as a "rowing oar" during the powerstroke, but the high degree of disorder is unexpected if the powerstroke is modeled as a simple swing of a stiff lever arm.

The nitroxide probes previously used to study the RLC were mobile on the protein surface and attached to a single site. An unfortunate probe orientation might render the probe insensitive to a change in the orientation of the neck. Alternately, the observed probe disorder could be the result of local disorder of the protein surface. Hence, we have placed a paramagnetic probe on a second site, Cys-108 of chicken gizzard regulatory light chain (RLC), which was exchanged for native RLC in skinned, glycerinated, rabbit psoas muscle fibers. Although this probe also shows some restricted motion relative to the protein, the amplitude of the motion is a little smaller than that found previously, and the probe's preferential orientation in rigor fibers is more along the fiber axis than that of the previous probe. In addition we were able to obtain far better results with labeled myosin subfragments, eliminating some ambiguities in the earlier work.

MATERIALS AND METHODS

Purification of Chicken Gizzard Regulatory Light Chain. Chicken gizzard myosin was purified by the method of Ikebe and Hartshorne (1985). Two modifications were made: (1) following the first addition of 1 M $MgCl_2$ to a final concentration of 150 mM, additional ATP was added to a final concentration of 5 mM; (2) before centrifugation for 3 h at 75 000*g*, ATP was added, again to a final concentration of 5 mM. Chicken gizzard RLC was isolated from gizzard myosin as follows: The final myosin pellet was dissolved in a minimum volume of 0.5 M KCl, 1 mM $NaHCO_3$, and 1 mM DTT at pH 7.5. The gizzard myosin (10−15 mg/mL) was denatured in 8 M urea, 50 mM Tris (pH 8), and 1 mM DTT and stirred for 2 h at room temperature. EtOH was added to a final concentration of 50%. Gizzard RLC was isolated with a 50%−70% EtOH precipitation, and the RLC pellet was brought up in a minimum volume and dialyzed against a binding buffer (0.12 M KAc, 20 mM MOPS, 5 mM $MgCl_2$, 1 mM EGTA, and pH 7.0). Purity of gizzard RLC was checked via SDS gel electrophoresis.

Paramagnetic Labeling of Gizzard RLC. Cys-108 of chicken gizzard RLC was labeled with a paramagnetic probe, 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy (IASL), which binds covalently to the reactive sulfhydryl. The gizzard RLC (1−2 mg/mL) was incubated in the binding buffer with addition of 0.2 mM IASL for 2 h at 0 °C. After the labeling reaction, the free IASL was eliminated using a small sizing column in binding buffer.

Exchange of Gizzard RLC for Rabbit RLC. Thin strips of rabbit psoas muscle, 1−2 mm in diameter, were dissected and chemically skinned, as described by Cooke et al. (1988). The skinned rabbit fibers were dissected into small bundles and incubated in an exchanging buffer (10 mM PO_4, 20 mM EDTA, 50 mM KCl, pH 7.0 at 35 °C) with additional 0.5−1 mg/mL of IASL-labeled gizzard RLC/mL for 2 h, with occasional shaking. Since this procedure may extract troponin C as well as the native RLC, after the extraction and exchange the exchanged fiber was washed for 10 min with the binding buffer, and additional troponin C (about 1 mg/mL) was added for 30 min on ice. About 80%−100% of the native RLC was exchanged for gizzard RLC as determined by gel electrophoresis.

EPR Spectroscopy. EPR measurements were performed with an ER/200D EPR spectrometer from Bruker Inc. (Billerica, MA). X-band, first-derivative absorption spectra were obtained with the following settings: microwave power, 25 mW; center field, 3475 gauss; time constant, 100 ms. The sweep time was 10 s, the modulation was 0.08−0.2 mT at a frequency of 100 kHz, and the total sweep width was 10 mT. About 100 exchanged fibers mounted in a flow cell (Zhao et al., 1995) were located in the center of a TE_{102} cavity. Each spectrum used in data analysis represented the average of 20−40 distinct sweeps over a single experimental preparation. Spectra taken for shorter times (5−10 sweeps) were similar except for poorer signal-to-noise ratios. Temperature was maintained by slowly blowing cooled nitrogen gas or hot air through the cavity and monitored by a small thermistor embedded in the flow cell adjacent to the fibers. EPR experiments were taken at 4 and 24 °C. Spectra were reproducible between experiments on individual preparations, indicating that fiber function was not greatly impaired by the experimental conditions. Spectra of relaxed myofibrils were obtained by flowing a relaxing solution past myofibrils that were immobilized by glass wool.

Spectral Simulations. The experimental spectra were compared to spectra simulated from a solution to the spin Hamiltonian using a program written by Dr. P. Fajer et al. (1990a). Two angles, θ and ϕ, define probe orientation relative to the fiber axis, with θ representing the angle between the probe's principal axis and the magnetic field

(the tilt angle) and ϕ representing the twist about this axis. The data did not permit accurate modeling of ϕ. The average twist angle, ϕ_0, was arbitrarily set at 45° and modeled as a Gaussian distribution with full width 10°. The presence of the component P2 (defined below and in Figure 2A) presented a problem, since it overlaps the low-field features of P1 (defined below), and a highly mobile probe population is not easily simulated. Because P2 was present with a magnitude that differed between preparations, an approximate pure component was generated from the difference between spectra from different preparations. An artificial spectrum based on these difference spectra, smoothed to reduce noise, was used in subsequent fits to data. Model spectra were generated to fit component P1 by variation of the angle of the principal axis to the magnetic field, θ_0, and of the distribution about this central angle. Fitting was carried out primarily on the spectra of fibers aligned parallel to the magnetic field, which are more sensitive to changes in tilt angle. Simulations of fibers aligned perpendicular to the field using the angular distribution obtained also fit the corresponding data.

Mechanical Measurement. Measurements of fiber mechanics were performed at both 4 and 24 °C, as described by Cooke et al. (1988) and Zhao et al. (1995).

Solutions. All experimental buffers maintained a constant calculated ionic strength of 230 mM. Rigor buffer contained 120 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 20 mM MOPS (pH 7.0). For relaxing solution, additional 5 mM MgATP, 30 mM CP, and 5 mg of CK/mL were added to the rigor buffer. Activation of fibers was induced by adding ~1.1 mM $CaCl_2$ into the relaxing solution (final pCa ≈ 4.5).

RESULTS

Rotational Mobility of the Probe. EPR spectra are capable of providing information on both the orientation and the rotational mobility of paramagnetic probes. A nitroxide spin probe produces a spectrum consisting of three lines or peaks, which are affected by motion and orientation of the probe. When the orientations of probes in the sample are random, the shape of the EPR spectrum is sensitive to rotational motion with correlation time (τ_c) in the range from 10^{-12} to 10^{-6} s. Figure 1 shows a series of EPR spectra of randomly oriented samples at low and high temperatures: free IASL in solution (Figure 1A), IASL−gizzard RLC in solution (Figure 1B), and randomly oriented labeled rigor fibers (Figure 1C). The solid and dashed lines were obtained at 24 and 4 °C, respectively. Temperature had little effect on the samples in solution, but there was a noticeable effect in randomly oriented fibers (Figure 1C). Both high- and low-field peaks broadened at lower temperature, indicating a shift to a more immobilized probe.

The free IASL and IASL-labeled gizzard RLC in solution are cases of rapid tumbling (10^{-12}−10^{-9} s). For this case, τ_c can be determined by the Lorentzian line width of the three peaks, which are related both to τ_c and to the anisotropy of the hyperfine interaction (Stone et al., 1965). The ratio of the line heights between the central peak and low-/high-field is increased as τ_c decreases. The correlation times were calculated from eq 5 in Stone et al. (1965). In the case of labeled RLC in solution (Figure 1B), the ratios of line heights of the central peaks to the peaks in the low- and high-field were 1.03 and 1.39, respectively. The width of the central peak-to-peak separation was 0.26 mT. The calculated τ_c from the equations of Stone et al. (1965) for the labeled light chain in solution was 6.6×10^{-10} s. The τ_c of free IASL (Figure 1A) obtained from a similar calculation was 6.0×10^{-11} s. The correlation time of the bound probe was much smaller than that expected for a protein of the size of RLC, $(5-8) \times 10^{-9}$ s. This indicates that the probe attached to a free light chain had a high degree of mobility relative to the protein.

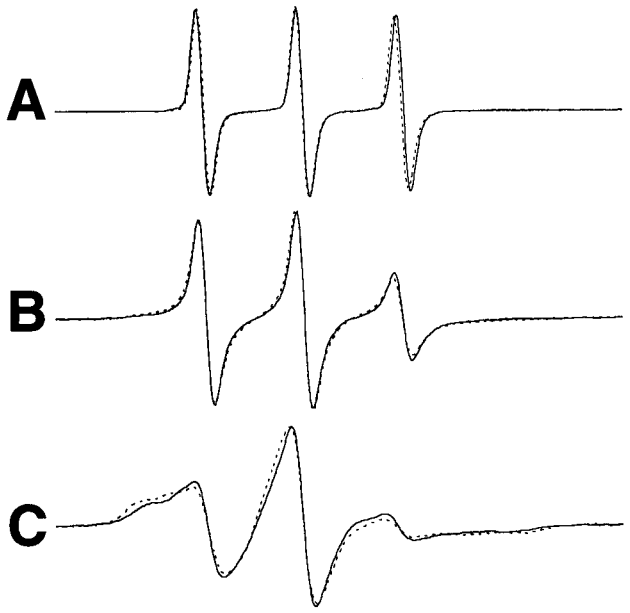

FIGURE 1: First-derivative X-band absorption EPR spectra of random IASL samples at 24 °C (solid line) and 4 °C (dashed). (A) 10 μM free IASL solution in 25 μL capillary. There was no difference between the spectra obtained at high and low temperatures. (B) 1 mg/mL IASL-labeled gizzard RLC solution in 25 μL capillary. The IASL probe was attached to Cys-108 on gizzard RLC. There was no difference between the spectra obtained at high and low temperatures. (C) Randomly oriented, labeled, rigor fibers in a flat cell. The base line for each spectrum was 10 mT.

When labeled RLC was exchanged onto myosin heads, the lines broadened, showing that the motion of the probe was restricted and/or slowed down. Figure 1C (solid line) shows the spectrum of randomly oriented fibers exchanged with IASL-gizzard RLC in rigor conditions at 24 °C. The spectrum of randomly oriented fibers in relaxing conditions was identical (data not shown). The spectrum was composed of two components, appearing as two overlapping peaks in the low-field region but better separated in the high-field region. These components arose from populations of probes with different degrees of motional freedom, possibly reflecting different modes of interaction of probes with proteins, different conformational states of the light chain or neck, or properly versus improperly bound light chains. At 4 °C (Figure 1C, dashed line) there were apparently three motional populations (notice the three overlapping peaks in the low-field region, corresponding to three better-separated features in the high-field region), including one midway between the two seen at 24 °C. This change could be an effect of the reduction in Brownian motion with temperature, but since Brownian motion depends on absolute temperature this is unlikely to account for a large change in the spectrum. More likely, the new population represents a conformational state of the protein stabilized by the lower temperature or a mode of probe−protein interaction likewise stabilized.

Interpretation of these spectra is model dependent. As described in the next section, the spectra of oriented rigor fibers showed that the probe motion was not isotropic but was spatially restricted. Assuming the probes were undergoing rapid (sub-nanosecond) motion within cone(s), the half-width of the cone can be estimated following the calculation of Griffith and Jost (1976). The measured values of splitting were compared to a rigid limit value obtained in the absence of motion. The rigid limit value for the splitting between high and low field was determined to be 6.8 mT at 24 °C by treating randomly oriented fibers with saturating ammonium sulfate (data not shown). The splittings between the high- and low-field peaks for the two populations in the random spectrum at 24 °C were 6.4 and 3.9 mT (Figure 1C, solid line). The calculated order parameters were 0.8 and 0.2, and the half-widths of the cones were 30° and 70°, respectively (Griffith & Jost, 1976). The relative contributions were difficult to determine, but the more mobile component contained more spins than the more immobile one. Due to the nearly isotropic nature of the more mobile component, the rotational correlation time could be estimated by the method of Stone et al. (1965) from the relative heights of the three lines. The peak heights were determined from an approximate spectrum for this component obtained from a difference spectrum, described in Materials and Methods. The estimated rotational correlation time of the probe ($<1-2$ ns) was much faster than the expected rotational motion of the light chain. Therefore this component arose from probes that were bound to, but not immobilized by, the protein and thus did not report the protein orientation. The less mobile component, on the other hand, was substantially restricted by the protein surface and did report the protein orientation. At 4 °C (Figure 1C, dashed line), the more mobile population (smaller splitting) decreased, and a new population with intermediate mobility was evident. Also, the splitting of the outermost component increased somewhat, indicating decreased mobility.

Orientation of Probes in Rigor Fibers. Figure 2 shows EPR spectra obtained from a bundle of labeled fibers in rigor. The fibers were mounted either parallel (solid line) or perpendicular (dashed line) to the static magnetic field. Two populations of probes were evident in the parallel spectrum at 24 °C (Figure 2A, solid line). One population, P1 (indicated in Figure 2A), was ordered, producing broad peaks seen at low and high field as well as some of the intensity of the central peak. The ordered signals were produced by probes attached to gizzard RLC Cys-108 bound properly on the myosin RLC binding site. Another population, P2 (indicated in Figure 2A), gave rise to the second low-field peak and first high-field peak and also contributed some intensity to both the first peak in the low field and to the central peak. P2 was not orientation-dependent and was similar to the more mobile component in the random spectrum (Figure 1C), suggesting that it arose from the more mobile probe population. The relative fraction of ordered and mobile probes was determined from double integrations of the spectral components associated with P1 and P2 described below. The ratio of intensity of P2 to P1 depended on the preparation, suggesting that P2 arose largely from light chains that were not correctly attached to RLC sites on myosin, rather than from a different conformation of the myosin neck region (contrast Figures 2A and 6A). P2 represented approximately 70% of the probes in Figure 2A and 30% in Figure 6A, two representative preparations. In the spectra of fibers in rigor aligned perpendicular to the magnetic field (Figure 2A, dashed line) the mobile component, P2, was little changed, while P1 moved inward and under P2 in the low field, forming a shoulder on the P2 peak. At 4 °C (Figure 2B) the spectra were similar; the splitting of P1 was slightly wider, and P2 was reduced or spread out.

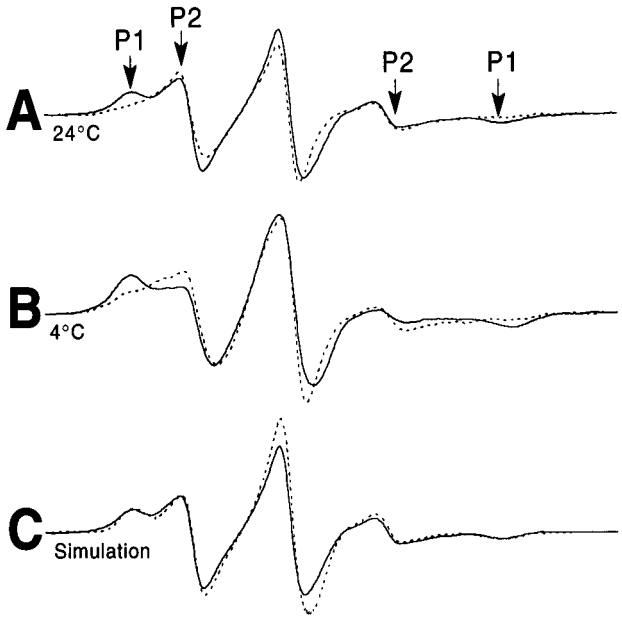

FIGURE 2: Effect of orientation of labeled rigor fibers on EPR spectra. The fibers were set in a flat cell and placed in the center of a TE_{102} cavity. The fibers were oriented parallel (solid line) and perpendicular (dashed line) to the magnetic field at (A) 24 °C and (B) 4 °C. P1 and P2 are defined in the text. Great differences between the parallel and perpendicular spectra indicate that the spin probes attached to the myosin neck were highly ordered in the rigor state. For the parallel spectra, the splittings between the low- and high-field peaks of P1 were 6.4 ± 0.1 mT and 6.7 ± 0.2 mT for 24 and 4 °C, respectively. (C) The spectrum of labeled fibers at 24 °C oriented parallel to the magnetic field (solid line, from A above) compared to a simulated spectrum (dashed line). The simulated spectrum is the sum of a simulated oriented component ($\theta_0 = 39° \pm 3°$, $\Delta\theta = 20° \pm 5°$), contributing to P1, and an empirical mobile component contributing to P2.

Two angles, θ and ϕ, define probe orientation relative to the fiber axis, with θ, the main determinant of the spectral shape, representing the angle between the probe's principal axis and the magnetic field. Spectra were simulated for P1 for different distributions of θ, added to the spectrum determined for P2, and compared to the data for fibers in rigor, as described in Materials and Methods. The best fits to the data were found for non-Gaussian distributions of angles, and the distributions were approximated as the sum of two or more Gaussian distributions. The spectral component P1 best fit an angular distribution centered at 39 ± 3° with a spread about this angle of 20 ± 5° full width at half-maximum (see Figure 2C). Errors were estimated by simulating a series of spectra and determining the range of values that produced equivalent fits to the data. An important caveat to this analysis is that probe mobility averages the hyperfine tensor values and narrows the spectra overall, an effect that is difficult to model exactly. Because we have used tensors that describe the spectrum of a rigid probe the absolute values of the probe distributions determined above are not exact. Because the mobility of the probes alters the

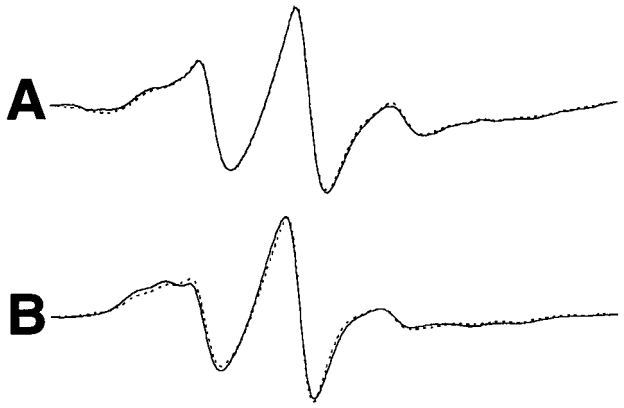

FIGURE 3: EPR spectra of the relaxed fibers at (A) 24 °C and (B) 4 °C. The fibers were oriented parallel (solid line) and perpendicular (dashed line) to the magnetic field. At 24 °C, the parallel and perpendicular spectra of the relaxed fibers were identical, indicating that the probes were disordered, but at 4 °C there were considerable differences between the parallel and perpendicular spectra, indicating that the probes were partially ordered.

tensors by approximately 10%, the perturbations produced in the analysis should also be about 10%. For the parallel spectra, the splittings between the peaks at low and high field were temperature dependent. The splitting was increased from 6.4 mT at 24 °C to 6.7 mT at 4 °C. This suggests that the lower temperature further restricted the motion of the oriented population of probes.

EPR Spectra of Relaxed Fibers. Spectra of fibers in relaxing conditions are presented in Figure 3 for fibers parallel (solid lines) or perpendicular (dashed lines) to the magnetic field. For the higher temperature (Figure 3A), parallel and perpendicular spectra were almost identical to each other, indicating that probes were highly disordered in this state. For the lower temperature (Figure 3B), a considerable difference between the parallel and perpendicular spectra was seen, indicating that the myosin neck region was partially ordered. The spectrum of randomly oriented, relaxed fibers was similar to that of randomly oriented fibers in rigor conditions (data not shown; see Figure 1C for rigor data). This indicates that the mobility of the neck was the same in rigor and relaxing conditions.

EPR Spectra of Active Fibers. Figure 4C shows EPR spectra of labeled fibers, generating active isometric tension, aligned parallel to the magnetic field. The spectra of active fibers were identical to those of relaxed fibers at both high and low temperatures. Figure 4D shows the difference spectra between relaxed and active spectra. These spectra were flat to within the experimental error. This indicates that the myosin neck region, in activation as in relaxation, was totally disordered at the higher temperature but partially ordered at the lower temperature. No novel angles were seen at either temperature in active fibers when compared to relaxed fibers.

EPR Spectra of Labeled Gizzard RLC-Exchanged S1/HMM Bound to Fibers in Rigor. The spectra of the labeled gizzard RLC-exchanged S1 (single myosin heads) bound to fibers in rigor at two different temperatures are shown in Figure 5. The spectra were quite similar to those of labeled fibers but with notable differences. Figure 6 shows the parallel spectra of fibers, HMM and S1 at 24 °C. The splitting between low- and high-field peaks of the oriented components was ~6.4 mT for labeled fibers and HMM and ~6.2 mT for S1; the splitting of the mobile component was ~3.9 mT for each preparation. The spectrum of HMM was essentially identical to that of the fibers, indicating that the interaction of two heads is sufficient to change the neck orientation from that of S1 to that of intact myosin bound to actin. Addition of MgATP in rigor buffer washed out both the ordered and mobile probes, demonstrating that the EPR signals arose from the labeled HMM or S1.

The spectra of the labeled gizzard RLC-exchanged S1 taken at 24 °C were fit in the same manner as spectra of labeled fibers, described above (data not shown). The orientation of the immobile component (corresponding to P1 in labeled fibers) was found to differ slightly from that of labeled endogenous myosin; it was equally well fit by a change in mean tilt angle, θ_0, from 39 ± 3° to 42 ± 3°, or by a change in the distribution about the mean tilt angle, $\Delta\theta$, from a full-width of 20 ± 5° to 15 ± 5°. The mobility of the probes was determined from the spectra of randomly oriented myofibrils, with the label either on endogenous

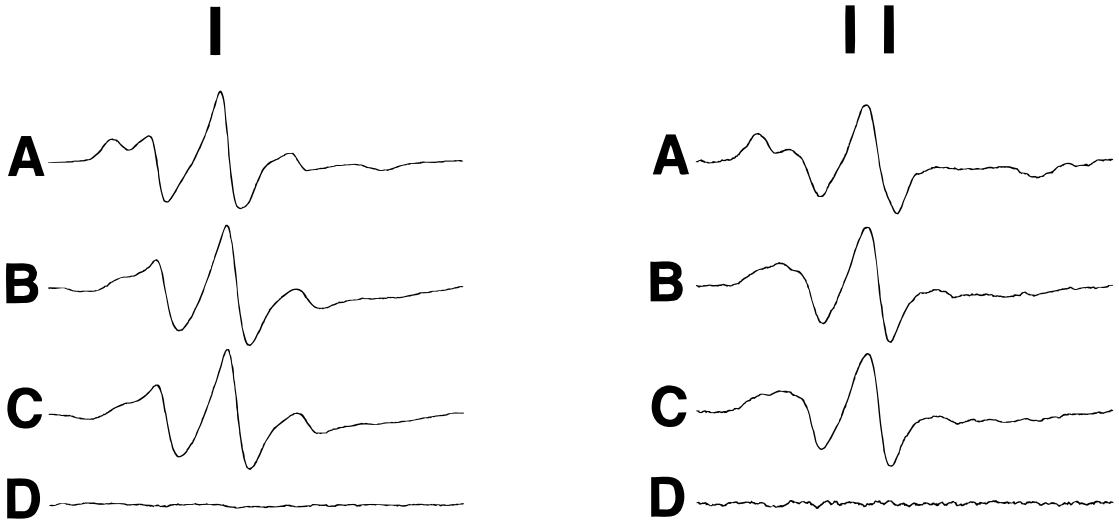

FIGURE 4: EPR spectra of fibers at (I) 24 °C and (II) 4 °C: (A) fibers in rigor; (B) relaxed fibers; (C) active fibers; (D) difference between active and relaxed spectra. The labeled fibers were placed in a capillary and aligned parallel to the magnetic field. The fibers were perfused with solutions producing rigor, relaxation, and active tension generation. The relaxed solution was obtained by adding 5 mM ATP, 30 mM CP, 5 mg of CK/mL into the rigor solution. Activating solution was obtained by adding ~1.2 mM $CaCl_2$ into the relaxing solution. The difference spectra (D) were flat within our experimental error, indicating no difference in the spectra of active and relaxed fibers.

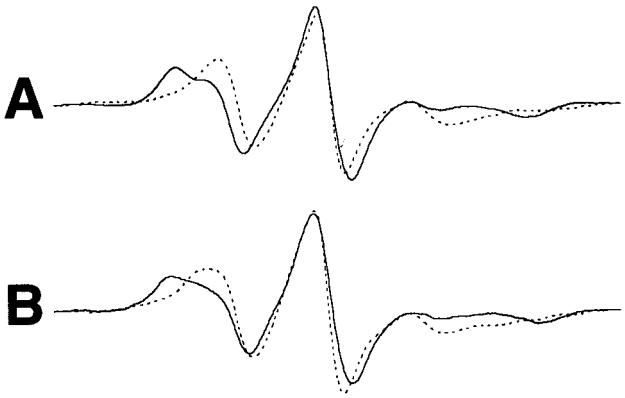

FIGURE 5: EPR spectra of unlabeled rigor fibers with labeled S1. IASL gizzard RLC was exchanged for rabbit native RLC in the proteolytic fragments, which were then diffused into skinned fibers. The spectra were taken at (A) 24 °C and (B) 4 °C. Fibers were aligned parallel (solid line) and perpendicular (dashed line) to the magnetic field. Washing the fibers with relaxing solution resulted in elimination of the signal.

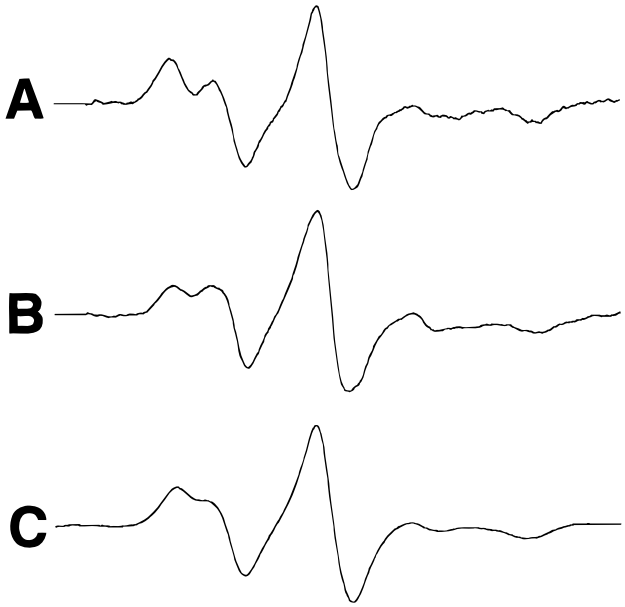

FIGURE 6: EPR spectra of (A) labeled rigor fibers; (B) unlabeled rigor fibers with labeled HMM; and (C) unlabeled rigor fibers with labeled S1. All spectra were taken at 24 °C with fibers aligned parallel to the magnetic field. The great similarity in the spectra indicates that the myosin neck was ordered primarily by the acto-myosin bond. The orientation of the neck is very similar in intact myosin and HMM, and only slightly different in S1, when bound to actin.

myosin or on S1 bound to unlabeled myofibrils. The splitting of the more immobilized component was the same for these two preparations, showing that the differences observed in the oriented samples did not arise from changes in probe mobility. The similarity in the spectra of endogenous myosin and S1 indicated that the major determinant of the orientation of the neck was the interaction of the myosin head with actin. However, the orientation of this region was changed slightly by the steric constraints associated with connecting the two heads together in HMM or in fibers.

Effect of Stress on Rigor Fibers. The atomic model of the acto-S1 complex suggests that the neck region may be flexible (Rayment et al., 1993a). This leads to the possibility that a passive force could alter the conformation of the neck region. Labeled muscle fibers in rigor buffer were mounted in a capillary that extended through a microwave cavity with fibers parallel to the magnetic field, see Cooke (1981). Fibers were well lined up so that external force could be homogeneously distributed inside the bundle of fibers. The EPR spectra were monitored as stress was applied to the fibers at room temperature. The stress was slowly increased up to 0.4 N/mm^2. No change was found in either the splitting or the line shape of the EPR spectrum during or after the loading process. We conclude that the orientation and motion of the probes were not affected by a steady external force.

Mechanics of Rabbit Muscle Fibers with Labeled Gizzard RLC. In a relaxing solution, the exchanged fibers achieved the normal relaxed tension and stiffness. Fiber isometric tension and stiffness were measured under isometric conditions. Active tension and stiffness developed by the exchanged fiber were about 90%−100% of control (unexchanged) values (data not shown). We conclude that the chicken gizzard RLC, bound to the neck of rabbit myosin, functions similarly to the native rabbit RLC in isometric contraction, and the exchange procedure does not impair fiber function. A similar result was found for exchange of gizzard RLC that carried a fluorescent probe (Ling et al., 1996).

DISCUSSION

A reasonable goal in the investigation of force production is to resolve a large rotation in the neck region of myosin that could produce the 5−10 nm displacement that is thought to occur during a powerstroke. This goal has been elusive. Although two recent structural studies have provided direct evidence that the myosin neck can undergo a rotation, neither study determined the exact change thought to occur between the beginning and the end of the powerstroke. The orientation of fluorescent probes attached to the RLC could be altered by application of a transient external force (Irving et al., 1995). This showed that a torque applied to the cross-bridge was able to alter the orientation of the neck. Similar experiments showed that the orientation of the catalytic domain did not undergo a change in orientation (Cooke, 1981). The change in orientation produced by the torque was found to be different in rigor fibers and in active fibers, suggesting that the average orientations of the neck were different in these two states. Although the fluorescent probes report changes in orientation of the neck occurring upon application of external forces, the changes in fluorescence are modest. The fluorescent probes are unable to provide the spatial resolution to resolve several populations of the probes, and so cannot answer the question of whether a small change in orientation has occurred in a majority of the myosin necks, or whether a larger change in orientation has occurred in a smaller subset of force generating cross-bridges. More recently the neck of a smooth muscle and a non-muscle S1 bound to actin has been observed by electron microscopy to rotate independently of the catalytic domain upon addition of ADP. The rotation was in a direction such that the release of ADP could provide at least a portion of a powerstroke (Jontes et al., 1995; Whittaker et al., 1995). However, it is unlikely that a major portion of the powerstroke occurs upon ADP release in skeletal muscle. These structural studies have been reinforced by functional studies of fragments of the myosin head. Here it has been found that myosin heads interacting with actin filaments are capable of generating force, eliminating other structures such as the myosin rod from playing a significant role in the force generation

process. Further deletions within the neck region have shown that as it becomes shorter, the velocity generated by the myosin fragments becomes smaller (Lowey & Trybus, 1994). Deletion of the entire neck produces a myosin fragment which moves actin at a very diminished velocity, suggesting that the ability of the catalytic region to generate motion by itself is very limited (Itakura et al., 1993; Uyeda, 1996). Addition of a third light chain increased the velocity, with a direct correlation between velocity and the length of the long α-helix that comprises the core of the neck region (Uyeda, 1996). These are the results that would be expected if the neck were acting as a lever.

These structural studies, together with the functional data obtained for myosins with necks of varying lengths, discussed above, show that the neck can rotate and that it appears to act like a lever in the generation of velocity. However, the exact angular distributions of the neck in the powerstroke remain undefined. Results obtained from spectroscopic probes in active fibers have not resolved a lever arm movement thought to occur during the powerstroke. Both paramagnetic and fluorescent probes placed on the RLC are ordered in rigor fibers but are almost completely disordered in both relaxed and active fibers (Hambly et al., 1992; Ling et al., 1996). Although the neck should have a fairly broad range of orientations if it is acting as a lever, complete disorder is unexpected. The presence of disorder could arise because of disorder of the RLC region or because of disorder of the probe relative to the protein surface. In addition, some probes may not be optimally oriented to detect a particular rotation. Some of the ambiguities cited above for the spectroscopic probes can be resolved by investigating spectra of probes attached to a different site. This helps eliminate questions of the interaction between the probe and the protein surface. In addition, probes pointing in different directions could be sensitive to motion that was missed by probes oriented in a single direction insensitive to the motion. In the present paper, we report results from paramagnetic probes attached to a new site on the myosin regulatory light chain. These probes are preferentially aligned at about 39° to the axis of the muscle filaments, while those investigated by Hambly and co-workers (1991, 1992) were oriented in a direction more perpendicular to the filament axis. The two different orientations can eliminate the possibility that one of the probes is oriented in a direction that is unfavorable to observe a particular motion.

The location of the IASL probe on myosin can be determined to a good approximation. In the crystal structure of myosin, the two light chains wrap around an 85 Å long α-helix that extends from the catalytic domain to the rod. RLC occupies approximately one-half of the helix adjacent to the junction with the rod (Rayment et al., 1993b). The spin probe is attached to Cys-108 of chicken gizzard RLC, which was exchanged for native RLC. The location of the probe can be inferred by analogy to the crystal structures of chicken skeletal myosin (Rayment et al., 1993a,b). Cys-108 of chicken gizzard RLC corresponds to Val-103 of chicken skeletal RLC, which is located close to the catalytic domain, facing the gap between light chains and on the same side as the nucleotide pocket. RLC structures that have been determined are very similar, suggesting that Cys-108 of gizzard RLC is located in a similar position (Rayment et al., 1993b; Xie et al., 1994). Thus the spin probe is positioned near the middle of the myosin neck region, a critical site for examining orientation and motion of the lever arm.

The effects of the attachment of the neck to the rod can be investigated by contrasting the spectra of rigor fibers with the spectra of labeled heavy meromyosin or S1, which are bound to unlabeled fibers. Here, we find the dramatic result that the spectra of HMM are identical to those of labeled myosin heads in the rigor fibers. In previous studies the spectra of the subfragments contained a large disordered component, which could have indicated a greater degree of freedom for the neck region in the absence of an attachment to the rod (Hambly et al., 1991). Our result shows that the attachment of the myosin head to the myosin rod has not induced a change in the relative orientations of this part of the neck. However, the orientation of the neck of S1 differs by several degrees from that of HMM and endogenous myosin, suggesting that a very limited distortion of the neck arises from the interaction of the two myosin heads when bound to actin. However, the observed distortion amounts to only a few degrees, much less than what might be expected from other studies. Due to the mismatch of the subunit repeats of the actin and myosin filaments, considerable distortion must occur in the proteins in order for all myosin heads to attach to actin in rigor fibers. Electron micrographs of rigor fibers have suggested that much of this distortion occurs in the neck of the myosin molecule, while spectroscopic probes have shown that it does not occur in the orientation of the catalytic domain (Taylor et al., 1984; Thomas & Cooke, 1980). Thus, it is not clear why a greater difference between S1 and fibers (or HMM) is not seen in the EPR spectra. There has been one report that spin probes attached to RLC show significantly different angles for S1 and for HMM (Arata, 1990). We do not have an explanation for the difference between these results and the those observed here. However, Hambly and co-workers (1991, 1992) also did not observe a significant difference between S1, HMM and fibers, although their spectra were not as unambiguous as those obtained here.

The results obtained at the lowered temperature, 4 °C, differ only slightly from those obtained at the higher temperature of 24 °C. The degree of order found in the rigor fibers decreases slightly, as indicated by broadening of the peaks in the EPR spectra, but more dramatically one now finds order in the relaxed fibers. These results are more in accord with numerous structural studies which have found ordered myosin heads in relaxed fibers. However, these studies indicate that order in relaxed rabbit fibers increases with temperature, whereas we observe increased order in relaxed fibers at lowered temperatures. In active fibers, the results at the lower temperature again reflect the same observations as those seen at the higher temperature. There are no new orientations introduced that were not observed in the relaxed fibers.

The results obtained at this second probe site largely support the results obtained by Hambly and co-workers from probes attached to the Cys-125 of the rabbit light chain (Hambly, 1991, 1992). The spectra show that the probes have a high degree of order in muscle fibers in rigor. We see no evidence for the existence of discrete binding angles for the two heads of a single myosin, but rather only a single orientation. An essentially isotropic spectrum was obtained in relaxed fibers. Of particular importance is the finding that the active fibers again show the same degree of disorder

as obtained in the relaxed fibers. Studies of probe mobility suggest that the disorder in the active fibers does not arise from a disordering of the probe relative to the protein surface. The mobility of the probe is not altered when the fibers go from the rigor state to the relaxed state. This lack of change in mobility suggests that the interaction between the probe and the protein surface has not been changed. If the probe is ordered on the protein surface in rigor, it most likely remains ordered in the relaxed and active states, and thus the observed disorder arises in large part from disorder of the protein. We conclude that the neck region of myosin is highly disordered in active fibers.

In summary, the current results extend, and largely agree with, those obtained previously for a spin probe attached to another site on the light chain. Although neither probe is optimal, e.g., both show some motion relative to the protein, the present probe is slightly more restricted and is oriented in a different direction. The general agreement of results from the two sites greatly strengthens the conclusions. The neck is ordered in rigor, and the order is determined largely by the bond with actin rather than by the connection to the rod. The neck is disordered in relaxed fibers, although some degree of order was seen here at lower temperatures. The neck is disordered in active fibers with a spectrum that is indistinguishable from relaxed fibers. This is the same result as was obtained by paramagnetic probes at a different site and by fluorescent probes at this same site (Hambly et al., 1992; Ling et al., 1996), suggesting that the observed disorder is not probe or location specific. Current models of force generation by myosin do not explain the almost isotropic disorder observed by probes attached to RLC in active fibers.

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REFERENCES

Arata, T. (1990) *J. Mol. Biol. 214*, 471−474.
Barnett, V. A., Fajer, P., Polnaszek, C. F., & D. D., T. (1986) *Biophys. J. 49*, 144−14.
Cooke, R. (1981) *Nature 294*, 570−1.
Cooke, R. (1986) *Crit. Rev. Biochem. 21*, 53−118.
Cooke, R., Crowder, M. S., & Thomas, D. D. (1982) *Nature 300*, 776−8.
Cooke, R., Franks, K., Luciani, G., & Pate, E. (1988) *J. Physiol. 395*, 77−97.
Fajer, P. G., Bennett, R. L. H., Polnaszek, C. F., Fajer, E. A., & Thomas, D. D. (1990a) *J. Magn. Reson. 88*, 111−125.
Fajer, P. G., Fajer, E. A., & Thomas, D. D. (1990b) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5538−42.
Goldman, Y. E. (1987) *Annu. Rev. Physiol. 49*, 632−654.
Griffith, O. H., & Jost, P. C. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 454−519, Academic Press, New York.
Hambly, B., Franks, K., & Cooke, R. (1991) *Biophys. J. 59*, 127−38.
Hambly, B., Franks, K., & Cooke, R. (1992) *Biophys. J. 63*, 1306−13.
Holmes, K. C., Popp, D., Gebhardt, W., & Kabsch, W. (1990) *Nature 347*, 44−49.
Ikebe, M., & Hartshorne, D. (1985) *J. Biol. Chem. 260*, 13146−13153.
Irving, M., Allen, T. S., Sabidodavid, C., Craik, J. S., Brandmeier, B., Kendrickjones, J., Corrie, J. E. T., Trentham, D. R., & Goldman, Y. E. (1995) *Nature 375*, 688−691.
Itakura, S., Yamakawa, H., Toyoshima, Y. Y., Ishijima, A., Kojima, T., Harada, Y., Yanagida, T., Wakabayashi, T., & Sutoh, K. (1993) *Biochem. Biophys. Res. Commun. 196*, 1504−1510.
Jontes, J. D., Wilsonkubalek, E. M., & Milligan, R. A. (1995) *Nature 378*, 751−753.
Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37−44.
Ling, N., Shrimpton, C., Sleep, J., Kendrickjones, J., & Irving, M. (1996) *Biophys. J. 70*, 1836−1846.
Lowey, S., & Trybus, K. M. (1994) *Biophys. J. 68*, 120s.
Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., & Milligan, R. A. (1993a) *Science 261*, 58−65.
Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Thomchick, D. R., Benning, M. M., Winkelman, D. A., Wesenberg, G., & Holden, H. M. (1993b) *Science 261*, 50−57.
Roopnarine, O., & Thomas, D. D. (1995) *Biophys. J. 68*, 1461−1471.
Stone, T. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010−1017.
Taylor, K. A., Reedy, M., C., Cordova, L., & Reedy, M. K. (1984) *Nature 310*, 285−291.
Thomas, D. D. (1987) *Annu. Rev. Physiol. 46*, 691−709.
Thomas, D. D., & Cooke, R. (1980) *Biophys. J. 32*, 891−906.
Uyeda, T., Abramson, P., Spudich, J. (1996) *Proc. Nat. Acad. Sci. U.S.A.* (in press).
Whittaker, M., Wilson-Kubalek, E., M., Smith, J., E., Faust, L., Milligan, R., M., & Sweeney, H., L. (1995) *Nature 378*, 748−751.
Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalabokis, V. N., Szent-Gyorgyl, A. G., & Cohen, C. (1994) *Nature 368*, 306 - 312.
Zhao, L., Pate, E., Baker, A., J., & Cooke, R. (1995) *Biophys. J. 69*, 994−999.

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