Steady-State Fluorescence Polarization Studies of the Orientation of Myosin Regulatory Light Chains in Single Skeletal Muscle Fibers Using Pure Isomers of Iodoacetamidotetramethylrhodamine

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ABSTRACT The regulatory light chain (RLC) from chicken gizzard myosin was covalently modified on cysteine 108 with either the 5- or 6-isomer of iodoacetamidotetramethylrhodamine (IATR). Labeled RLCs were purified by fast protein liquid chromatography and characterized by reverse-phase high-performance liquid chromatography (HPLC), tryptic digestion, and electrospray mass spectrometry. Labeled RLCs were exchanged into the native myosin heads of single skinned fibers from rabbit psoas muscle, and the ATR dipole orientations were determined by fluorescence polarization. The 5- and 6-ATR dipoles had distinct orientations, and model orientational distributions suggest that they are more than 20° apart in rigor. In the rigor-to-relaxed transition (sarcomere length 2.4 μm, 10°C), the 5-ATR dipole became more perpendicular to the fiber axis, but the 6-ATR dipole became more parallel. This orientation change was absent at sarcomere length 4.0 μm, where overlap between myosin and actin filaments is abolished. When the temperature of relaxed fibers was raised to 30°C, the 6-ATR dipoles became more parallel to the fiber axis and less ordered; when ionic strength was lowered from 160 mM to 20 mM (5°C), the 6-ATR dipoles became more perpendicular to the fiber axis and more ordered. In active contraction (10°C), the orientational distribution of the probe dipoles was similar but not identical to that in relaxation, and was not a linear combination of the orientational distributions in relaxation and rigor.

INTRODUCTION

Muscle contraction is thought to be driven by a conformational change in the head domain of myosin. Relatively small conformational changes near the nucleotide binding site may be amplified by the elongated light chain region acting as a lever to produce nm-scale displacements of the filaments (Vibert and Cohen, 1988; Rayment et al., 1993a,b; Holmes, 1997). This mechanism predicts that large changes in the orientation of the light chain region would occur during the cycle of ATP hydrolysis and force generation by actomyosin.

The orientation of this region has been studied in skeletal muscle fibers by attaching a dipole probe to the myosin regulatory light chain (RLC) and exchanging the labeled RLC into a demembranated fiber in a low [Mg] solution (Hambly et al., 1991, 1992; Irving et al., 1995; Ling et al., 1996; Allen et al., 1996). This approach showed that the orientation of the light chain region depends on the contractile state of the fiber. Using fluorescence polarization with rhodamine probes, changes in the orientation can be followed in single muscle fibers on the submillisecond time scale and correlated with force transients (Irving et al., 1995; Allen et al., 1996). A large change in orientation follows flash-photolytic release of ATP in rigor fibers, accompanying either ATP binding to myosin or the subsequent detachment of myosin from actin (Allen et al., 1996). A smaller orientation change occurs during a length step applied to an actively contracting fiber, and continues during the rapid force recovery that is thought to signal the working stroke in the myosin head (Irving et al., 1995).

These fluorescence polarization studies used iodoacetamidotetramethylrhodamine (IATR) to label the single cysteine (Cys^{108}) of chicken gizzard RLC. Much of the work used IATR from Molecular Probes (Eugene, OR), although widely divergent results were obtained with different batches of this product (Sabido-David et al., 1994). There are two isomers of IATR, with the iodoacetamido group at either the 5- or 6-position (Fig. 1), so the variability of the results may have been related to variations in isomer composition. Pure isomers of 5- and 6-IATR have been synthesized (Corrie and Craik, 1994), and 6-IATR was used to label RLC for the rapid length step experiments described above (Irving et al., 1995). The 5- and 6-isomers of IATR have been used to label the reactive thiol of the myosin heavy chain in isolated skeletal muscle fibers (Ajtai et al., 1992; Berger et al., 1996). Fluorescence polarization measurements from these probes suggested that this region of the heavy chain does not change its orientation during the working stroke (Berger et al., 1996).

Here we report the purification and characterization of chicken gizzard RLC labeled at Cys^{108} with 5- and 6-IATR, and steady-state fluorescence polarization ratios obtained from muscle fibers into which the labeled RLCs had been exchanged. The labeled RLCs will be referred to as 5- and

Received for publication 10 September 1997 and in final form 18 March 1998.

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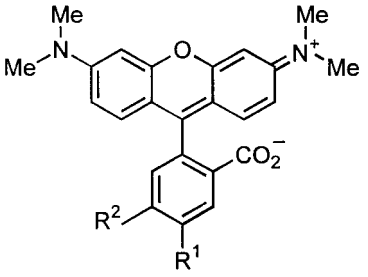

5-IATR $R^1 = ICH_2CONH$, $R^2 = H$

6-IATR $R^1 = H$, $R^2 = ICH_2CONH$

FIGURE 1 Structure of the 5- and 6-isomers of IATR

6-ATR-RLC, because the iodine of IATR is displaced in the labeling reaction. The two ATR-RLC isomers gave very different results: for example, in rigor fibers the 6-ATR dipole shows a preference for orientations perpendicular to the fiber axis, whereas the 5-ATR dipole shows a preference for parallel orientations. Preliminary results from these steady-state measurements have been presented in abstract form (Sabido-David et al., 1994). The following paper (Hopkins et al., 1998) describes transient changes in the orientation of the ATR-RLC probes in response to rapid length steps and photolysis of caged ATP.

MATERIALS AND METHODS

Preparation of ATR-labeled regulatory light chains

Chicken gizzard regulatory light chain expressed in *Escherichia coli* was the wild-type sequence with an added N-terminal glycine (Rowe and Kendrick-Jones, 1992); this was the kind gift of Dr. J. Kendrick-Jones. It was stored at −20°C at a protein concentration of up to 50 mg/ml in 25% sucrose, 25 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 8.0. This RLC has a single cysteine residue (Cys^{108}) that can be specifically labeled with iodoacetamido derivatives, and binds tightly and specifically to the skeletal myosin heavy chain (Trybus and Chatman, 1993). Pure 5- or 6-isomers of IATR were synthesized as described by Corrie and Craik (1994). RLC was diluted to 50 μM in labeling buffer, containing 20 mM potassium phosphate (KP_i), 100 mM potassium propionate (KPr), 1 mM $MgCl_2$, pH 7.4, incubated with 6 M guanidine hydrochloride and 10 mM DTT for 1 h at room temperature to reduce any disulfides formed during storage and to disperse aggregates, then dialyzed at 5°C into labeling buffer. Some batches of RLC tended to precipitate during dialysis, and for these batches the dialysis step was replaced by passage through a Sephadex G-25 column (PD10; Pharmacia) preequilibrated with labeling buffer. 5- or 6-IATR (final concentration 200 μM), from a 15 mM stock solution in dimethylformamide, was added to RLC in labeling buffer and incubated in the dark for 2 h at room temperature. The reaction was stopped by the addition of 10 mM sodium 2-mercaptoethanesulfonate (MESNA). Unconjugated dye was separated from RLC by passage through a PD10 column preequilibrated with extract buffer (maximum 2.5 ml labeling solution per column) containing 20 mM EDTA, 50 mM KPr, 10 mM KP_i, pH 7.0. The extent of RLC labeling was measured by reverse-phase HPLC (Hichrom VYDAC 218TP54 C18 column with guard column 218GCC54), eluted with a linear gradient (1 ml/min) formed from 0.1% trifluoroacetic acid (TFA) in H_2O and 0.082% TFA in acetonitrile. The gradient ran from 40% to 60% acetonitrile in 20 min, and labeled and unlabeled RLC typically eluted at ~54% and ~55% acetonitrile, respectively (Fig. 2). Protein elution was monitored by absorbance at 215 nm, and rhodamine fluorescence with excitation at 549 nm and emission above 580 nm. The product of the reaction between RLC and 5- or 6-IATR (Fig. 2, *A* and *B*) showed an unknown leading peak, accounting for 5–8% of the protein, unlabeled RLC (10–20% of total protein), and RLC that had been covalently modified with IATR (~80%). This preparation of ATR-labeled RLC is referred to subsequently as unpurified ATR-RLC.

A more homogeneous preparation, referred to subsequently as purified ATR-RLC, was prepared as follows. An aliquot of the stock solution of RLC was passed down a PD10 column that had been preequilibrated in labeling buffer. The eluted protein was diluted to 50 μM with labeling buffer, and 5- or 6-IATR was added to a final concentration of 300 μM. The mixture was incubated as described above, then quenched by the addition of 10 mM MESNA. The quenched solution was gel-filtered, using PD10 columns equilibrated in fast protein liquid chromatography (FPLC) buffer (10 mM KP_i, 50 mM KPr, and 1 mM $MgCl_2$, pH 7.0). The recovered protein, typically 0.8 mg/ml, was purified by FPLC on a Mono-S HR 5/5 cation exchange column (Pharmacia). RLC (3.5–5 mg) in FPLC buffer was loaded onto the column and eluted with a gradient of 150–300 mM KCl in FPLC buffer, with a total elution volume of 40 ml and a flow rate of 1 ml/min. Protein elution and rhodamine fluorescence were monitored as described above for HPLC. The main peak fractions, eluting at ~200 mM KCl, were 95% pure by HPLC analysis (Fig. 2, *C* and *D*). These fractions

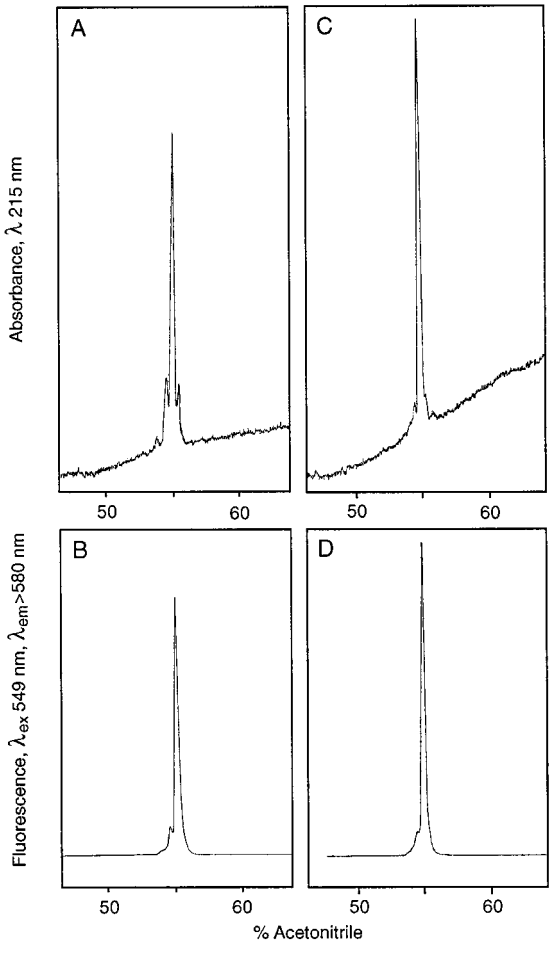

FIGURE 2 Analytical reverse-phase HPLC traces of gizzard regulatory light chain labeled with 6-iodoacetamidotetramethylrhodamine. (*A* and *B*) Absorbance and fluorescence, respectively, for the labeled protein before purification by FPLC. (*C* and *D*) Corresponding traces after FPLC purification.

were combined and their RLC concentration estimated using an extinction coefficient for the rhodamine chromophore of 52,000 M^{-1} cm^{-1} at 528 nm (Corrie and Craik, 1994), under the assumption that the rhodamine:RLC ratio was 1:1. The combined fractions were placed in a dialysis bag, which was surrounded with solid sucrose and kept at 4°C. The sucrose was renewed each hour until the volume inside the dialysis bag was reduced by the factor (typically 7–10) required to bring the estimated protein concentration to 1.5 mg/ml. This generally took 6–7 h. Finally the concentrated protein was passed through a PD10 column as above, and stored at −80°C after snap freezing in liquid nitrogen. The final concentration of labeled RLC in extract buffer as used for exchange into muscle fibers was 34–50 μM, calculated from the rhodamine absorbance as described above.

The specificity of RLC labeling was assessed after FPLC purification by electrospray mass spectrometry (VG Platform instrument). The intact labeled protein showed one predominant component with measured mass 20214 and 20216 Da for 5- and 6-ATR-RLC, respectively. The calculated mass of ATR-RLC is 20212 Da. The 6-ATR-RLC sample showed a minor component (~5%) with a mass of 20657 Da, similar to the expected value (20653 Da) for RLC labeled with two ATR groups.

To confirm the site specificity of labeling, each labeled RLC (15 μM) was digested with 15 mg/ml trypsin (Lorn Laboratories) for 4.5 h at 37°C in 0.67 M guanidine hydrochloride, 42 mM NH_4HCO_3. In each case a single fluorescent peptide was isolated from the unlabeled peptides using the HPLC column described above, eluted with a gradient of 10–60% acetonitrile over 50 min at a flow rate of 1 ml/min. The labeled peptide eluted at ~43% acetonitrile. The predicted tryptic peptide containing Cys^{108} extends from Asn^{104} to Arg^{123}. The ATR-labeled peptide has the expected composition $C_{127}H_{165}N_{31}O_{37}S$, corresponding to a mass of 2750 Da. The value measured by electrospray mass spectrometry was 2749 Da for both the 5- and 6-ATR-labeled peptides.

Muscle fibers and solutions

New Zealand white rabbits (3.5–5.5 kg) were killed by sodium pentobarbitone injection (200 mg/kg). The psoas muscles were exposed and cooled by filling the peritoneal cavity and surrounding the back area with crushed ice (Kawai et al., 1993), and irrigated with a relaxing solution (fiber preparation and skinning (FPS) solution), containing 70 mM KPr, 8 mM Mg acetate, 5 mM potassium EGTA, 7 mM sodium ATP, 6 mM imidazole, 1 mM sodium azide, pH 7.0 with 0.1 mM phenylmethylsulfonyl fluoride (Sigma, Poole, Dorset, England), 0.1 mg/ml soybean trypsin inhibitor (Sigma), and 8 μg/ml leupeptin (Sigma) to inhibit protease activity. Fiber bundles (~40 mm long and ~2 mm diameter) were tied to wooden sticks at the in situ length with surgical silk thread and excised from the psoas muscle. After a 1-h incubation in FPS solution at 0°C, the bundles were transferred to FPS solution plus 0.5% Triton X-100 (BDH, Poole), stirred for 1 h at 0°C, then washed three times for 30 min in FPS solution. The bundles were glycerinated by overnight incubation at 5°C in FPS solution containing 25% glycerol, then stored for up to 3 weeks at −20°C in FPS solution containing 50% glycerol.

Immediately before an experiment, a segment of a single fiber, 3–4 mm long, was dissected from a bundle in the storage solution on a microscope stage cooled to 5°C. Aluminum T-clips were crimped at each end of the segment (Goldman and Simmons, 1984), and the fiber was transferred to a 40-μl glass trough containing standard relaxing solution (Table 1). The T-clips were attached at one end to a fixed hook and at the other end to a force transducer (AE801; Aksjeselskapet Mikro-elektronikk, Horten, Norway). Sarcomere length and fiber diameter were measured using a 32× objective (Leitz L32, N.A. 0.40) and ocular micrometer, and hook separation was adjusted to set sarcomere length to 2.4 μm. On the microscope stage were five 40-μl glass troughs that could be rotated to change fiber solutions (Sleep, 1990). The temperature of the trough was measured with a 400-μm-diameter thermistor (Thermometrics, Edison, NJ) near the fiber, and controlled to ± 0.1°C within the range 0–30°C by varying the flow of cold nitrogen or hot air onto the lower surface of the copper plate carrying the troughs. The temperature could be switched between 5°C and 30°C in less than 1 min.

Labeled RLC was exchanged into single muscle fibers by a procedure slightly modified from that of Ling et al. (1996). The solution compositions are given in Table 1. The fiber was transferred to a 0.1 mM ATP ("prerigor") relaxing solution for 3 min, to Mg rigor solution for 3 min, to the EDTA rigor "extract" solution for 3 min, then to extract solution containing the labeled RLC and 10 mM fresh DTT, all at 10°C. The temperature was raised to 30°C for 30 min to allow RLC exchange, then decreased to 10°C. The fiber was washed in relaxing solution for 3 min, then incubated sequentially in relaxing solutions containing 0.5 mg/ml rabbit muscle troponin (Sigma) for 10 min, and 0.5 mg/ml troponin C (Potter, 1982; a kind gift of Dr. T. St. C. Allen) for 5 min to replace these proteins lost during RLC exchange.

Fluorescence polarization

Steady-state fluorescence polarization ratios were measured using a modified epifluorescence microscope as described by Ling et al. (1996). The light source was a HBO 100-W mercury lamp. The excitation light passed through a 540 nm (30 nm FWHM) interference filter and polarizing prism, then was reflected by the 580-nm dichroic mirror/filter assembly (Zeiss filter set 487714) through a 32× objective lens (Leitz 32×, N.A. 0.40) to illuminate a 48-μm-diameter spot at the fiber with light polarized either parallel or perpendicular to the longitudinal fiber axis. Fluorescent light was collected by the same objective lens, passed through the dichroic mirror/filter assembly (barrier filter 590 nm, long-pass), a circular aperture in the primary image plane that coincided with the image of the spot on the fiber, and a Wollaston beam-splitting prism that separated it into components polarized parallel and perpendicular to the fiber axis. The intensities of these components were measured with two photomultipliers. An aperture at the back of the objective limited the N.A. of the excitation and fluorescent light to 0.25. At this aperture the depolarization produced by the objective is negligible (Wilson and Mendelson, 1983), and no correction was applied for this effect.

Four fluorescence intensities were measured: ${}_{\parallel}I_{\parallel}$, ${}_{\parallel}I_{\perp}$, ${}_{\perp}I_{\parallel}$, ${}_{\perp}I_{\perp}$, where ${}_{m}I_{n}$ subscripts denote the fluorescence intensity for exciting light of polarization m and emitted light of polarization n, and $\parallel$ and $\perp$ refer to linear polarizations parallel and perpendicular to the fiber axis. These intensities

TABLE 1 Solution compositions

Solution	Imidazole	K_2EGTA	Na_2ATP	Na_2CP	Mg acetate	CaEGTA	KPr	free [Mg^{2+}]	I.S.
Relaxing (160 mM I.S.)	25.0	5.0	5.0	5.0	7.0	0.0	80.0	1.8	159
Relaxing (20 mM I.S.)	7.0	2.0	1.1	0.0	2.1	0.0	0.7	1.0	20
Prerigor	25.0	7.2	0.1	5.0	1.5	0.0	91.6	1.5	150
Rigor	10.0	2.5	0.0	0.0	2.2	0.0	130.0	2.0	151
Preactivating	25.0	0.20	5.0	5.0	7.0	0.0	90.0	2.1	155
Activating	25.0	0.0	5.0	10.0	7.0	10.0	40.0	1.9	149

Creatine kinase (1 mg/ml) was added to the preactivating and activating solutions. CP, creatine phosphate; Pr, propionate; I.S., ionic strength. The pH was 7.0. Values are in mM.

were used to calculate four polarization ratios:

$$P_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\parallel}I_{\perp})/({}_{\parallel}I_{\parallel} + {}_{\parallel}I_{\perp})$$

$$P_{\perp} = ({}_{\perp}I_{\perp} - {}_{\perp}I_{\parallel})/({}_{\perp}I_{\perp} + {}_{\perp}I_{\parallel})$$

$$Q_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\perp}I_{\parallel})/({}_{\parallel}I_{\parallel} + {}_{\perp}I_{\parallel})$$

$$Q_{\perp} = ({}_{\perp}I_{\perp} - {}_{\parallel}I_{\perp})/({}_{\perp}I_{\perp} + {}_{\parallel}I_{\perp})$$

RESULTS

5- and 6-isomers of ATR on RLC have distinct orientations in muscle fibers

Either 5- or 6-ATR-RLC was exchanged into rabbit psoas muscle fibers, and the fluorescence polarization ratios $P_{\parallel}$, $P_{\perp}$, $Q_{\parallel}$, and $Q_{\perp}$ measured in relaxation, active contraction, and rigor (Table 2). $P_{\parallel}$ and $P_{\perp}$ depend on the angle between the ATR emission dipole and the fiber axis, and $Q_{\parallel}$ and $Q_{\perp}$ depend on the angle between the absorption dipole and the fiber axis. Because the emission and absorption dipoles of ATR are almost colinear (Chen and Bowman, 1965), the corresponding P and Q ratios are similar (Ling et al., 1996). The present results will be presented in terms of P ratios; the observed changes in Q ratios were consistent with those in P ratios, except where noted. A higher value of $P_{\parallel}$ and a lower value of $P_{\perp}$ indicate that the ATR dipole is more parallel to the fiber axis.

The P ratios obtained with 5-ATR were significantly different from those with 6-ATR in all conditions studied (Table 2, Fig. 3). The difference was largest in rigor; here the 5-ATR dipole had a preference for orientations parallel to the fiber axis ($P_{\parallel}$ was larger than $P_{\perp}$), whereas the 6-ATR dipole had a preference for orientations perpendicular to the fiber axis ($P_{\perp}$ was larger than $P_{\parallel}$). In relaxation and active contraction, 6-ATR had a stronger perpendicular preference than 5-ATR.

The observed P ratios were fitted using two models for the orientational distribution of the probes. In both models the probe dipoles are assumed to move within a cone of semiangle δ on a time scale that is rapid compared to the fluorescence lifetime (Irving, 1996). In the Gaussian model (Allen et al., 1996; Ling et al., 1996), the center axis of the cone has a Gaussian orientation distribution with peak angle θ_g with respect to the fiber axis and dispersion σ. In the helix plus isotropic model (Tregear and Mendelson, 1975), a fraction f of the cone axes are isotropically distributed, and the remainder are at an angle θ_h to the fiber axis. δ was measured in the accompanying paper (Hopkins et al., 1998). Its value is the same in relaxation, active contraction, and rigor, and is 20° for 6-ATR-RLC and 25° for 5-ATR-RLC.

In rigor the peak of the Gaussian distribution (θ_g) and the angle of the ordered fraction in the helix plus isotropic model (θ_h) were more than 20° larger for 6-ATR than for 5-ATR (Table 2). Because the fluorescence polarization ratios are sensitive to the axial but not the azimuthal component of the three-dimensional angle between the 5- and 6-ATR dipoles, 20° is a lower limit for the three-dimensional angle. In relaxation and active contraction, the axial offset between the 5- and 6-ATR dipoles was smaller than in rigor (Table 2), and θ_g and θ_h were 5–8° larger for 6- than for 5-ATR.

A similar set of measurements was made with the unpurified preparations of 5- and 6-ATR-RLC (see Materials and Methods). The Gaussian dispersion (σ) and the isotropic fraction of probes (f) were consistently larger for the unpurified than for the purified preparation, for both isomers and in either relaxation, active contraction or rigor. The differences were particularly large for 6-ATR. For example,

TABLE 2 Polarization ratios and fitted orientation parameters for IATR-labeled RLC

	n	$P_{\parallel}$	$Q_{\parallel}$	$P_{\perp}$	$Q_{\perp}$	θ_g	σ	θ_h	f
A. 5-ATR									
Relaxed	6	0.323	0.340	0.348	0.330	49.0	26.5	55.3	0.811
		(0.001)	(0.004)	(0.001)	(0.004)	(0.1)	(0.1)	(0.1)	(0.001)
Active	6	0.310	0.336	0.359	0.334	50.1	26.4	57.7	0.791
		(0.004)	(0.010)	(0.002)	(0.006)	(0.3)	(0.5)	(0.4)	(0.010)
Rigor	6	0.388	0.398	0.297	0.286	41.6	29.1	41.7	0.822
		(0.003)	(0.005)	(0.003)	(0.005)	(0.4)	(0.3)	(0.5)	(0.005)
B. 6-ATR									
Relaxed	11	0.259	0.279	0.431	0.405	55.8	22.7	62.8	0.623
		(0.003)	(0.003)	(0.003)	(0.010)	(0.2)	(0.3)	(0.3)	(0.005)
Active	11	0.233	0.259	0.436	0.414	56.9	21.7	63.1	0.575
		(0.009)	(0.013)	(0.009)	(0.008)	(0.5)	(0.7)	(0.6)	(0.014)
Rigor	9	0.060	0.112	0.539	0.501	64.8	18.5	67.9	0.332
		(0.006)	(0.006)	(0.004)	(0.004)	(0.3)	(0.2)	(0.2)	(0.007)

Mean fluorescence polarization ratios at sarcomere length 2.4 μm, 10°C, and the mean of the orientational distribution parameters determined by fitting the P ratios from each fiber. The values in parentheses indicate the standard error of the mean, for the number of fibers shown in the column headed n. θ_g and σ are the peak angle and dispersion in the Gaussian model; θ_h and f are the ordered angle and fraction of isotropic probes in the helix plus isotropic model. θ_g, θ_h, and σ are in degrees. The semiangle of the cone in which the dipoles move on a time scale faster than the fluorescence lifetime (δ) is 25° for 5-ATR and 20° for 6-ATR (Hopkins et al., 1998).

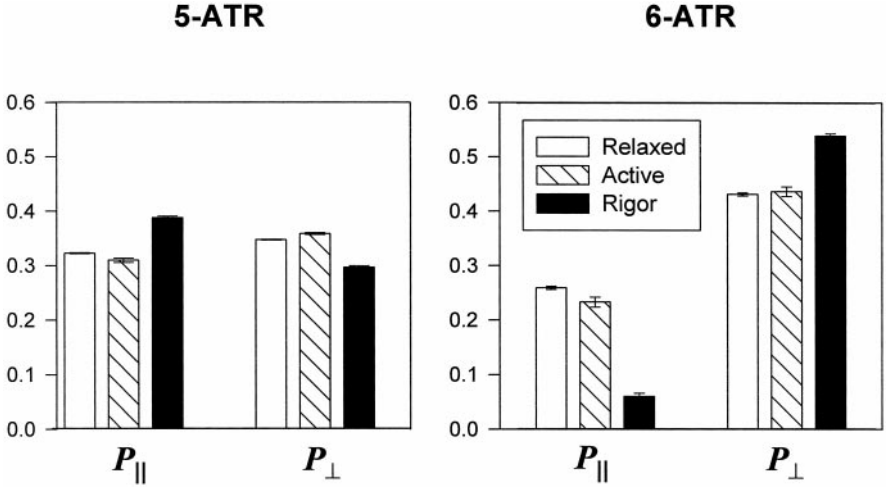

FIGURE 3 Polarization ratios $P_{\parallel}$ and $P_{\perp}$ from fibers containing 5- and 6-ATR-RLC in relaxation, active contraction, and rigor (sarcomere length 2.4 μm, 10°C). Values (mean ± SE) were taken from Table 2.

in relaxed fibers, σ for the unpurified preparation of 6-ATR-RLC was 38.9 ± 1.8° (mean ± SE, eight fibers), almost double the value of 22.7 ± 0.3° observed for the purified fraction (Table 2).

Comparison of probe orientations in relaxation, rigor, and active contraction

For both 5- and 6-ATR, the polarization ratios observed in rigor differed from those in either relaxation or active contraction (Table 2 and Fig. 3), as reported previously for mixed isomer IATR on RLC (Ling et al., 1996; Allen et al., 1996). The small differences between P ratios in relaxation and active contraction were statistically significant at the 5% level for both $P_{\parallel}$ and $P_{\perp}$ with 5-ATR and for $P_{\parallel}$ with 6-ATR (Table 2). This effect was not clearly resolved in the Q ratios, which have larger variability. In both the Gaussian and helix plus isotropic orientation models, the axial angles θ_g and θ_h differed by less than 3°. Thus the present results confirm the previous conclusion that the orientational distributions of the light-chain region of the myosin heads in relaxation and active contraction are similar but not identical (Ling et al., 1996; Allen et al., 1996).

The large changes in polarization ratios between rigor on the one hand, and relaxation and active contraction on the other, were in opposite directions for 5- and 6-ATR. In rigor the 5-ATR dipole became more parallel to the fiber axis, but the 6-ATR dipole became more perpendicular to the fiber axis. θ_g and θ_h decreased by 7–14° for 5-ATR and increased by 5–8°, respectively, for 6-ATR (Table 2). These movements were accompanied by relatively small changes in the Gaussian dispersion (σ) for either isomer. The isotropic fraction of probes, f, in the helix plus isotropic model, was substantially lower in rigor for 6- but not for 5-ATR.

Effect of filament overlap on the orientation of 6-ATR-RLC in relaxation and rigor

The change in RLC conformation between relaxation and rigor depends on binding of the myosin head to actin. Relaxed fibers containing 6-ATR-RLC were stretched to a sarcomere length of 4.0 μm to eliminate overlap between actin and myosin filaments. The stretch produced a small reversible decrease in $P_{\perp}$ (Fig. 4), corresponding to a small decrease in the axial angle of the probes. θ_g decreased by ~2° and θ_h by ~4° (Table 3). When relaxed fibers were put

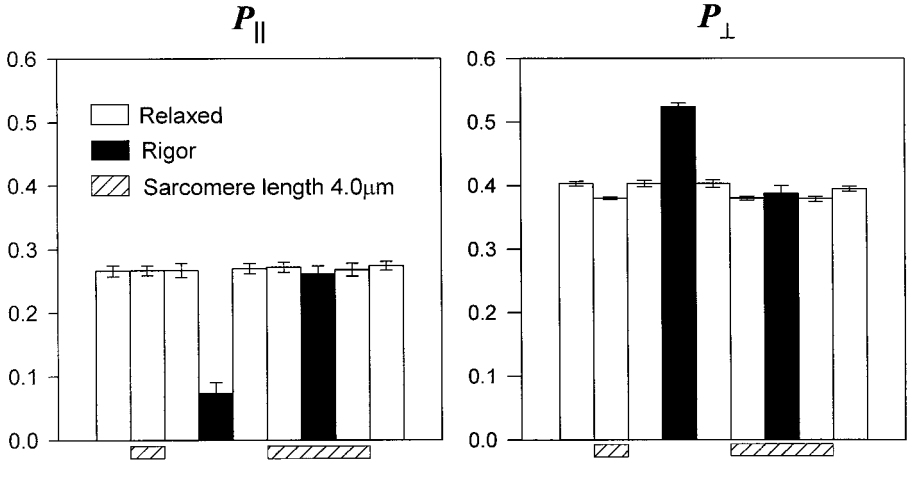

FIGURE 4 Polarization ratios $P_{\parallel}$ and $P_{\perp}$ (mean ± SE, n = 6) in relaxation (*white bars*) and rigor (*black bars*) in fibers containing 6-ATR-RLC at full filament overlap (sarcomere length (s.l.), 2.4 μm) and no filament overlap (s.l., 4.0 μm, marked by the *shaded bars* under the histograms). Temperature, 10°C. The measurements were made in the sequence from left to right in each panel.

TABLE 3 Dependence of polarization ratios and fitted orientation parameters on ionic strength and sarcomere length in relaxing solution

Ionic strength (mM)	Sarcomere length (μm)	$P_\parallel$	$P_\perp$	θ_g	σ	θ_h	f
160	2.4	0.265	0.423	55.2	22.6	62.2	0.636
		(0.009)	(0.006)	(0.5)	(0.3)	(0.5)	(0.016)
20	2.4	0.187	0.463	59.2	20.5	64.8	0.501
		(0.010)	(0.004)	(0.4)	(0.3)	(0.2)	(0.014)
160	4.0	0.273	0.378	53.6	20.8	58.7	0.639
		(0.006)	(0.009)	(0.1)	(0.8)	(0.6)	(0.016)
20	4.0	0.259	0.388	54.3	20.4	59.5	0.618
		(0.009)	(0.004)	(0.3)	(0.6)	(0.3)	(0.018)

Mean polarization ratios (5°C) from fibers containing 6-ATR-RLC, and the mean of the orientational distribution parameters determined by fitting the P ratios from each fiber with $\delta = 20°$. The values in parentheses indicate the standard error of the mean ($n = 7$). θ_g and σ are the peak angle and dispersion in the Gaussian model; θ_h and f are the ordered angle and fraction of isotropic probes in the helix plus isotropic model. θ_g, θ_h, and σ are in degrees.

into rigor at a sarcomere length of 4.0 μm, there was no significant change in either $P_\parallel$ or $P_\perp$ (Fig. 4), showing that the large changes seen at short sarcomere length (2.4 μm) were due to binding of myosin to actin. These results confirm those obtained by Ling et al. (1996), using unpurified RLC labeled with mixed isomer IATR. The orientation of probes on the reactive thiol of the myosin heavy chain also remains constant during the relaxed-to-rigor transition at a sarcomere length of 4.0 μm (Nihei et al., 1974).

Effect of varying temperature and ionic strength in relaxed muscle fibers

When the temperature of relaxed fibers from rabbit psoas muscle is raised from 12°C to 17°C, the helical order of the myosin heads increases (Wray, 1987; Lowy et al., 1991), and the heads become more parallel to the filament axis (Folkes et al., 1996). In relaxed fibers containing 6-ATR-RLC, $P_\parallel$ and $P_\perp$ were 0.269 ± 0.006 (mean ± S.E., $n = 7$) and 0.419 ± 0.004, respectively, at 5°C. The corresponding values at 30°C were 0.321 ± 0.004 and 0.367 ± 0.004. Thus the 6-ATR dipoles became more parallel to the fiber axis as the temperature was raised; θ_g decreased by ~6° and θ_h by ~4°. This was accompanied by an increase in the fraction of isotropic probes (f) in the helix plus isotropic model, but no clear change in the dispersion (σ) in the Gaussian model.

In low ionic strength relaxing solution, myosin heads are weakly bound to actin, in a rapid equilibrium between bound and unbound states (Brenner et al., 1982; Schoenberg, 1988). It has been suggested that this weakly bound state might correspond to the intermediate preceding the force-generating transition or working stroke in active contraction (Eisenberg and Hill, 1985), with the rigor state corresponding to the end of the working stroke. We therefore investigated the conformation of the relaxed state at low ionic strength with the 6-ATR-RLC probe, for comparison with the results from active contraction and rigor.

In relaxing solution at 20 mM ionic strength (sarcomere length 2.4 μm, 5°C), $P_\parallel$ was lower and $P_\perp$ higher than in the standard 160 mM ionic strength relaxing solution (Table 3). θ_g increased by ~4° and θ_h by ~2.5° at low ionic strength. There was a small decrease in the Gaussian dispersion (σ), and a larger decrease in the fraction of isotropic probes (f). These effects were mediated by binding of myosin heads to actin; when the overlap between actin- and myosin-containing filaments was abolished by stretching relaxed fibers to a sarcomere length of 4.0 μm, there was no significant effect of lowering the ionic strength to 20 mM (Table 3; values of $P_\parallel$ and $P_\perp$ at 20 and 160 mM ionic strength, sarcomere length 4.0 μm, are not significantly different at the 5% level).

The changes in probe orientation associated with lowering ionic strength at a sarcomere length of 2.4 μm were not due to the accompanying compression of the filament lattice. Osmotic compression by the addition of 6% Dextran T-500 (Pharmacia) in 160 mM ionic strength relaxing solution produced a decrease in fiber diameter of 17.8 ± 0.4% (mean ± SE, $n = 6$), with a concomitant increase in $P_\parallel$ (from 0.258 ± 0.016 to 0.276 ± 0.013) and a decrease in $P_\perp$ (from 0.432 ± 0.007 to 0.407 ± 0.003). These changes in polarization ratios are in the direction opposite to those produced by lowering the ionic strength.

The changes in probe orientation produced by weak binding of myosin heads to actin in low ionic strength relaxing solutions are in the same direction as, but smaller than, those produced by strong binding in rigor. To test whether the probe orientational distribution in 20 mM ionic strength relaxing solution could be expressed as a linear combination of that in 160 mM ionic strength relaxing solution and that in rigor, we calculated fluorescence intensities ${}_mI_n$ from the polarization ratios using the normalization function $({}_\parallel I_\parallel + 2{}_\parallel I_\perp + 2{}_\perp I_\parallel + 8{}_\perp I_\perp/3)$. This weighted sum of the polarized intensities is independent of dipole orientation for an immobile helical set of probes with colinear excitation and emission dipoles (equations 1–4 of Tregear and Mendelson, 1975), and remains a good approximation in the presence of the amount of rapid probe motion observed here (equations 31, 34, 36, and 42 of Irving, 1996). The parameter $F = [{}_mI_n$ (Relaxed, 20 mM I.S.) $- {}_mI_n$ (Relaxed, 160 mM I.S.)]/[${}_mI_n$ (Rigor) $- {}_mI_n$ (Relaxed, 160 mM I.S.)] should correspond to the fraction of probes in the rigor orientation during

relaxation at 20 mM ionic strength (I.S.). The values of F for $_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$, and $_{\perp}I_{\perp}$ calculated from the P ratios were 0.51 ± 0.02, 0.47 ± 0.04, and 0.50 ± 0.03 (mean ± SE, $n = 5$), respectively. The F values calculated from the corresponding Q ratios were in the range 0.58–0.66. Similar calculations based on Q ratios from 6-ATR-RLC measured on a different set-up in relaxing solutions of ionic strength 20 mM and 200 mM and in rigor gave F values in the range 0.7–0.8 (Hopkins et al., 1998). Both sets of measurements suggest that the orientational distribution of the RLC region of the myosin heads in relaxing solution at 20 mM ionic strength cannot be distinguished, by steady-state polarization ratio measurements, from a linear combination of the orientational distribution in relaxing solution at 160–200 mM ionic strength with that in rigor. The fraction of heads in the rigor orientation is likely to be in the range 0.5–0.8.

DISCUSSION

Use of RLCs labeled with defined IATR isomers to study myosin orientation in muscle fibers

Many previous studies have used extrinsic probes to investigate changes in the orientation of myosin heads in muscle fibers. Ideally such probes would constitute a single molecular species attached to a single specific site on the myosin head, so that the measured orientation parameters would correspond to a homogeneous population of probes. Such specificity is difficult to achieve by labeling the intact sarcomere. However, the regulatory light chain (RLC) of myosin can be labeled as an isolated protein and subsequently exchanged into the sarcomere. Fluorescence micrographs of RLC-exchanged myofibrils show a bright A-band with a dark line at the position of the bare zone of the thick filaments, indicating specific incorporation of the labeled RLC into myosin heads (Ling et al., 1996).

Another limitation of some previous studies has been the purity and homogeneity of the probe molecule itself. Previous experiments with rhodamine probes have used IATR of unknown isomer composition and purity (e.g., Burghardt et al., 1983; Tanner et al., 1992; Ling et al., 1996; Allen et al., 1996). The 5- and 6- isomers of IATR have been used to label whole muscle fibers for fluorescence anisotropy studies (Ajtai et al., 1992; Berger et al., 1996). However, these two studies produced substantially different results with respect to both labeling specificity and measured fluorescence polarization parameters, probably because of differences in the purity of the IATR preparations used. Pure 5- and 6-IATR react predominantly with the reactive thiol of the myosin heavy chain in muscle fibers, and the orientation of the probe dipole in the fiber was similar for these two isomers (Berger et al., 1996). These results emphasize the importance of using well-characterized probe molecules for fluorescence polarization studies.

In the present experiments we labeled Cys^{108} of chicken gizzard RLC with pure 5- and 6-IATR (Corrie and Craik, 1994), and purified the labeled RLCs by FPLC. Mass spectrometry and tryptic digestion identified the purified materials as the expected ATR-RLC conjugates. HPLC analysis indicated that the purification step removed contaminating species representing ~20% of the total RLC in the unpurified preparation (Fig. 2). The labeled RLCs were exchanged into demembranated muscle fibers for measurements of probe orientation by fluorescence polarization, and the ATR dipoles were found to be substantially more ordered for the purified than the unpurified preparation in all conditions studied.

The 5- and 6-ATR dipoles take up different orientations on the RLC in a muscle fiber (Table 2). The difference is clearest in rigor; here a comparison of the parallel and perpendicular polarization ratios indicates that the 5-ATR dipole has a preference for orientations parallel to the fiber axis, whereas the 6-ATR dipole has a preference for perpendicular orientations. Fitting the polarization ratios with Gaussian distributions of the dipole orientations with respect to the fiber axis showed that the peak angles (θ_g) of the distributions for 5- and 6-ATR differ by more than 20°. Because azimuthal offsets would not be detected by the present measurements, the three-dimensional angle between the 5- and 6-ATR dipoles is likely to be considerably larger than 20°.

A large angle between the dipoles is consistent with attachment of the iodoacetamido group to either the 5- or 6-positions of the pendant six-membered ring in the rhodamine structure (Fig. 1). If the fluorophore orientation were determined entirely by the position of the acetamido linkage to the RLC, the three-dimensional angle between the 5- and 6-ATR dipoles would be 60°. Rotation about single bonds within the acetamido linkage is likely to allow the conformation of the dipole to be affected by the interaction between the rhodamine derivative and the surface of the RLC, so the angle between the 5- and 6-ATR dipoles may depend on the protein and the site of attachment.

In previous experiments in which mixed isomer IATR was used to label RLC, varying results were obtained with different commercial batches of the reagent (Sabido-David et al., 1994), and this was probably related to varying isomeric composition. Ling et al. (1996) and Allen et al. (1996) labeled RLC with IATR batches 9B and 10A from Molecular Probes (Eugene, OR), and found Gaussian peak angles in rigor of 59° and 63°, respectively. These values are intermediate between those reported here for 5- and 6-ATR-RLC, 42° and 65°, respectively (Table 2), but are closer to the latter. The batches of IATR used by Ling et al. (1996) and Allen et al. (1996) probably contained mainly 6-ATR, with some 5-ATR also present.

Use of defined 5- and 6-IATR isomers and purified labeled RLC has improved the angular resolution of the fluorescence polarization technique; the probe orientational distributions in muscle fibers are more ordered. For example, the Gaussian dispersion (σ) for mixed isomer ATR-RLC in relaxed fibers was 30.5° (Ling et al., 1996) and 37.2° (Allen et al., 1996), compared with 24.2° and 22.7°

for 5- and 6-ATR-RLC in the present work (Table 2). This improvement is significant for precise measurements of the small changes in probe orientation seen in some experiments of physiological interest, for example when fast length changes are imposed on active muscle fibers (Irving et al., 1995).

Probe and RLC orientation in relaxation, rigor, and active contraction

The orientation of the RLC region of the myosin heads in rigor muscle fibers is distinct from that in either relaxation or active contraction (Table 2 and Fig. 3). The sarcomere length dependence of the relaxed-rigor difference shows that it is due to actin binding (Fig. 4). These results confirm the qualitative conclusions of earlier work with mixed isomer IATR (Ling et al., 1996; Allen et al., 1996), and the general implications of these results in relation to those obtained by other structural methods have been discussed therein.

One striking new finding is that the 5- and 6-ATR dipoles move in opposite directions with respect to the fiber axis in the transition from relaxation to rigor. For 6-ATR, θ_g is ~9° larger in rigor than in relaxation, but for 5-ATR θ_g is ~7° smaller in rigor (Table 2). Assuming that the 5- and 6-ATR dipoles maintain their orientation with respect to the coordinate frame of the RLC region during the transition from relaxation to rigor, this result places significant limits on the nature of the orientation change in the RLC region. In general, such an orientation change can be described by three angular parameters: an axial angle ("tilt") between some reference axis in the RLC region, conveniently taken as its long axis, and the fiber axis; an azimuthal angle, denoting the orientation of the RLC reference axis projected onto the equatorial plane (the plane perpendicular to the filament axis); and a torsional angle ("twist"), denoting rotation of the RLC region around its reference axis. Because of the cylindrical symmetry of the muscle fiber, the observed fluorescence polarization ratios are insensitive to the azimuthal angle, but are determined solely by the tilt and twist angles (Tregear and Mendelson, 1975). Tilting of the RLC region would tilt the 5- and 6-ATR dipoles in the same direction, unless they were on opposite sides of the equatorial plane, but the similar direction of the changes in polarization ratios from 5- and 6-ATR produced by applying small stretches to rigor fibers (Hopkins et al., 1998) suggests that the 5- and 6-ATR dipoles are on the same side of the equatorial plane. On the other hand, twisting of the RLC region could readily explain the opposite changes in θ_g for 5- and 6-ATR in the transition between relaxation and rigor. Further evidence for twisting of the RLC has been provided by the length step responses of the 5- and 6-ATR-RLC probes (Hopkins et al., 1998), and by fluorescence polarization data from bifunctional ATR derivatives (Corrie et al., 1997; Hopkins et al., 1997).

The observed polarization ratios and fitted orientation parameters from 5- and 6-ATR probes on the RLC were similar but not identical in relaxation and during active contraction (Table 2 and Fig. 3). EPR studies using probes on the reactive thiol (SH-1) of the myosin heavy chain have suggested that the orientation of the catalytic domain of the myosin head during active contraction could be represented as a linear combination of the relaxed and rigor orientations, with 10–20% of the probes in the rigor orientation (Cooke et al., 1982; Roopnarine and Thomas, 1995). On the other hand, the orientational distributions of EPR probes on the RLC during relaxation and active contraction were found to be indistinguishable (Hambly et al., 1992). In the previous fluorescence polarization studies in which RLC was labeled with mixed isomer IATR, there were small differences in polarization ratios between relaxation and active contraction (Ling et al., 1996; Allen et al., 1996), but it was not clear whether this was due to a small fraction of probes in the rigor orientation during active contraction. The present results exclude this possibility: the small changes in the P ratios from 5-ATR between relaxation and active contraction are in the opposite direction to the changes between relaxation and rigor (Fig. 3). Therefore these results suggest that the small difference in polarization ratios between relaxation and active contraction is due to a population of myosin heads in which the orientation of the RLC region is distinct from that in either relaxation or rigor.

The orientation of the RLC region in relaxed muscle fibers depends on the temperature, and this may be related to the changes in myosin head conformation with temperature in rabbit psoas fibers that have been detected by other techniques (Wray, 1987; Lowy et al., 1991; Folkes et al., 1996). However, the x-ray diffraction studies (Wray, 1987; Lowy et al., 1991) showed that the helical order of the myosin heads increases with temperature, whereas the ATR probes showed no evidence for an increase in orientational order with temperature. The cause of this apparent discrepancy is unknown.

In low ionic strength (20 mM) relaxing solution, the orientational distribution of the RLC region could be represented as a linear combination of those in relaxing solution of 160–200 mM ionic strength and in rigor (Table 3 here; table 2 of Hopkins et al., 1998). The fraction of probes in the rigor orientation was in the range 50–80%. This fraction is similar to the estimate of the fraction of myosin heads that are attached to actin in relaxing solution at 20 mM ionic strength based on stiffness measurements, ~50% (Brenner et al., 1982; Schoenberg, 1988). The effect of ionic strength on the orientation of the RLC region in relaxed fibers is greater than that observed with EPR probes on SH-1 (Fajer et al., 1991); the EPR spectrum in low ionic strength (30 mM) relaxing solution could be represented as a combination of 83% of the spectrum in normal ionic strength relaxing solution (170 mM) and 17% of a spectrum corresponding to an orientational distribution centered on the rigor angle, but with greater orientational disorder.

SUMMARY

The orientation of the RLC region of the myosin heads in relaxed, contracting, and rigor muscle was investigated by fluorescence polarization, using pure 5- and 6-isomers of IATR to label the RLC, and exchanging purified ATR-labeled RLCs into single muscle fibers. The change in orientation of the RLC region caused by binding of the myosin heads to actin in rigor, inferred from previous fluorescence polarization measurements using mixed isomer IATR (Ling et al., 1996), was confirmed by the present experiments. The improved angular resolution achieved with purified 5- and 6-ATR-RLC also revealed a population of myosin heads in active contraction in which the orientation of the RLC region was distinct from that in either relaxation or rigor. Changes in orientation of the RLC region associated with variations in the temperature and ionic strength of the relaxing solution were also characterized. The importance of using pure isomer probes was shown most dramatically by the observation that the 5- and 6-ATR-RLC probes tilt in opposite directions with respect to the fiber axis in the transition between the rigor and the relaxed or active states, and this is likely to be due to twisting of the RLC region about its long axis during this transition. The following paper (Hopkins et al., 1998) follows up these steady-state experiments, employing the same probes to study transient changes in the orientation of the RLC region produced by rapid length steps and by the photolytic release of ATP.

We thank T. Rutherford and K. Eason for skilled technical assistance, Dr. S. A. Howell for mass spectral analysis, and Drs. Y. E. Goldman and S. C. Hopkins for helpful discussion.

This work was supported by the Wellcome Trust and Medical Research Council, U.K.

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