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Effect of negative mechanical stress on the orientation of myosin cross-bridges in muscle fibers

(fluorescence/polarization/rhodamine/rotation)

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ABSTRACT **The effect of positive and negative stress on myosin cross-bridge orientation in glycerinated muscle fibers was investigated by using fluorescence polarization spectroscopy of the emission from the covalent label tetramethylrhodamine-5-(and -6)-iodoacetamide (IATR) specifically modifying sulfhydryl one (SH1) on the myosin heavy chain. Positive tension was applied by stretching the fiber in rigor. Negative tension was applied in two steps by using a protocol introduced by Goldman *et al.* [Goldman, Y. E., McCray, J. A. & Vallette, D. P. (1988) *J. Physiol. (London)* 398, 75P]: relaxing a fiber at resting length and stretching it until the relaxed tension is appreciable and then placing the fiber in rigor and releasing the tension onto the rigor cross-bridges. We found, as have others, that positive tension has no effect on the fluorescence polarization spectrum from the SH1-bound probe, indicating that the cross-bridge does not rotate under these conditions. Negative tension, however, causes a change in the fluorescence polarization spectrum that indicates a probe rotation. The changes in the polarization spectrum from negative stress are partially reversed by the subsequent application of positive stress. It appears that negative tension strains the cross-bridge, or the cross-bridge domain containing SH1, and causes it to rotate.**

It was suggested some time ago that the translation of an actin filament relative to a myosin filament that occurs in active muscle contraction results from summated repetitive impulsions that myosin cross-bridges deliver to the adjacent actin filament (1, 2). Because a cross-bridge appears to be quasi-rigid when interacting with actin (3), its motion during impulsion would, in the simplest case, be a rotation. Therefore many experiments, particularly those using probes that report their direction while attached to the myosin cross-bridge, have sought to track active cross-bridges, looking for rotary motion. Several of us have concluded that the expected rotation does occur (4–7), but there are also those who have detected nothing (8–10), so the issue lingers unresolved.

Evidence for cross-bridge rotation need not come only from observations of active cross-bridges. If cross-bridges rotate, driven by their on-board ATPase, then the attitude of a cross-bridge corresponding to the completion of a hydrolysis and an impulsion should be the attitude in rigor. Therefore, if in applying an external force we stretch a fiber in rigor, we might expect to rotate the cross-bridges in the reverse direction. This experiment was attempted long ago by dos Remedios *et al.* (and many times since by others) (11–13, 24), but they saw no reorientation of the cross-bridges. Plausibly (because there are likely to be elastic segments in the subfragment 2 portion of myosin that connects the cross-bridge to the myosin filament) these authors attributed their negative result to the presence of a compliance between cross-bridge and filament. But skeptics have been quick to point out that a negative result could also arise because cross-bridges do not rotate in contraction.

If the dos Remedios type of experiment (rigor "pull") is frustrated by a compliance, the reverse experiment (rigor "push"), in which cross-bridges are pushed deeper into rigor, may still reveal reorientation, provided the compliance is incompressible. Until now, however, no one has thought of a way of pushing the cross-bridges beyond rigor. Recently, a protocol for doing so was introduced by Goldman *et al.* (14). In this work we employ their protocol to see whether cross-bridges can be passively rotated by compression.

The experimental protocol for the application of negative stress, shown in Fig. 1, consisted of two steps: a fiber in relaxation was stretched until there was an appreciable relaxed tension (a stretch of 10% will do this) and then placed in rigor. Then the ends of the fiber were released. The relaxed tension, originating possibly from stretching the myofibrillar proteins titin and/or nebulin (15, 25, 26), is released onto the rigor cross-bridges producing a negative stress. Goldman *et al.* (14) showed that rigor cross-bridges could support the negative stress. We investigated the effect of positive and negative stress on cross-bridge orientation by using the SH1 probe tetramethylrhodamine-5-(and -6)-iodoacetamide (IATR) and fluorescence polarization spectroscopy to detect probe orientation changes (7).

IATR specifically modifies the cross-bridge at SH1 in glycerinated muscle fibers (4), is not independently mobile on the cross-bridge (5), and does not impair fiber contractility (16). We investigated the orientation of the probe by using fluorescence polarization spectroscopy wherein the fluorescence polarization is measured as a function of the wavelength of the exciting light (7). The orientation of the absorption dipole of the probe varies with excitation wavelength. Therefore, recording the fluorescence polarization as a function of the exciting wavelength investigates the anisotropic angular degrees of freedom of the cross-bridge by effectively rotating the probe dipole within the cross-bridge.

We find that, in agreement with previous work, positive stress on an IATR-labeled cross-bridge in rigor does not induce cross-bridge rotation. Fibers at resting length pulled in increments of 0.5% of their length until breaking showed identical fluorescence polarization spectra over the entire wavelength scan. Labeled rigor cross-bridges under negative stress, however, have a different polarization spectrum from unstressed cross-bridges. The changes in the polarization spectrum indicate that the negatively stressed cross-bridge is rotated relative to the unstressed cross-bridge.

We estimated the fraction of the cross-bridges that were out of overlap with actin in the rigor push experiments due to the initial 10% stretch in relaxation to induce relaxed tension. We also measured the polarization spectrum from rigor cross-bridges at no overlap. With this information we ascer-

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Abbreviation: IATR, tetramethylrhodamine-5-(and -6)-iodoacetamide.

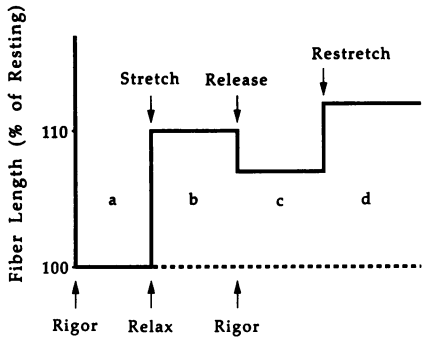

FIG. 1. Protocol for the application of negative stress to rigor cross-bridges. Fibers at resting length in rigor (part a) are mounted on the fiber stretching instrument shown in Fig. 4. The fibers are relaxed and stretched 10–20% of their resting length (part b) and then returned to the rigor bath. The negative relaxed tension is released onto the rigor cross-bridges (part c). The fiber is restretched in rigor (part d).

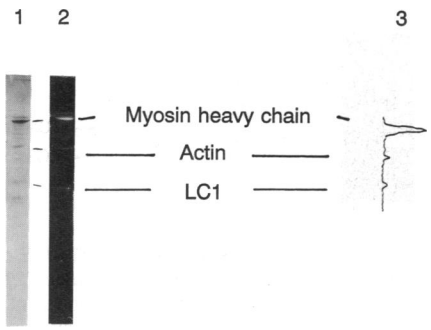

FIG. 2. Quantitative distribution and localization of IATR incorporation in muscle fiber proteins. Lane 1, Coomassie-stained SDS-PAGE gel of fiber extract; lane 2, same as lane 1 under UV illumination. Column 3, fluorescence scan of lane 2 indicating 85% of the intensity on the heavy chain, 8% in actin, and 7% in light chain 1 (LC1).

tained that there was no significant effect on the polarization spectrum from negatively stressed cross-bridges due to the small fraction of cross-bridges at no overlap.

We excluded the possibility that stressing the cross-bridge, either positively or negatively, causes a change in the actin filament order. This was done by measuring the polarization spectrum from IATR-labeled myosin subfragment 1 (S1) decorating fibers under positive and negative stress. In this experiment the exogenous IATR-S1 is a probe of actin filament order. We found that the polarization spectrum was unchanged by the imposition of positive or negative stress, indicating that these mechanical manipulations of the fibers do not cause any direct disordering of the fiber structure.

MATERIALS AND METHODS

Chemicals. ATP, trypsin, and chymotrypsin were from Sigma. The fluorescent probe IATR was purchased from Molecular Probes. All chemicals were analytical grade.

Solutions. Rigor solution contained 80 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 5 mM potassium phosphate, and 1 mM dithiothreitol at pH 7. Relaxing solution was rigor solution with 4 mM MgATP.

Preparation and Labeling of Muscle Fibers. We obtained rabbit psoas muscle fibers as described in ref. 4 and stored them in relaxing solution containing 50% (vol/vol) glycerol at −15°C for up to several weeks. The preparation of fibers for labeling was as in ref. 6. We labeled the skinned fibers with 160 μM IATR in relaxing solution without dithiothreitol for 30 min at 4°C with intensive stirring. The reaction was stopped with 1 mM dithiothreitol, and the excess of the dye was washed out with relaxing solution. Estimation of the labeling intensity and specificity was carried out either on the homogenate of the whole fiber or on purified isolated proteins from the fiber. To study the distribution of the label among the fiber proteins whole labeled fibers were homogenized and incubated in SDS sample buffer while stirring for 2 hr at room temperature. The homogenate was pelleted in a centrifuge and the supernatant was applied to a 15% SDS/PAGE slab gel. The electrophoretogram was analyzed either by visualizing the labeled proteins by their fluorescence or by staining the myofibrillar proteins with Coomassie brilliant blue. The parallel analysis of the two pictures was used to identify the labeled proteins or protein fragments. The quantitative distribution of the dye was measured by scanning the fluorescence of the unstained electrophoretogram as shown in Fig. 2. The intensity distribution shows that, in agreement with previous work (4), the probe was specific for the myosin heavy chain such that ≈85%, ≈7.9%, and ≈7.1% of the intensity was in the fluorescent bands corresponding to the myosin heavy chain, actin, and light chain 1, respectively. The more detailed specificity studies were done on myosin and S1 purified from the labeled fibers.

Proteins. Myosin was prepared from labeled glycerinated muscle fibers by using the method of Crowder and Cooke (17) with slight modification. The fibers were first washed with 5 mM Tris·HCl/1 mM EGTA, pH 8, and then homogenized in the myosin extracting solution containing 0.6 NaCl, 50 mM Tris·HCl, 5 mM sodium pyrophosphate, 5 mM $MgCl_2$, and 1 mM EGTA at pH 8. The extraction was carried out on ice for 3 hr with stirring. The homogenate was separated by centrifugation, and the supernatant containing the extracted myosin was collected. Myosin was purified by low ionic strength precipitation, and subsequently S1 was prepared according to the procedure of Weeds and Taylor (18). The fluorescence dye incorporation was estimated from the absorption spectra of the labeled proteins. Myosin and S1 concentrations were calculated using absorption coefficients at 280 nm of A(myosin) = 0.54 and A(S1) = 0.77 for a protein concentration of 1 mg/ml and a path length of 1 cm. Bound IATR was determined from the absorption spectrum using the molar extinction coefficient at 557 nm of $\varepsilon = 3.7 \times 10^4$ $(M \cdot cm)^{-1}$. The mean incorporation (±SD) of IATR in fibers was 0.42 ± 0.05 IATR per cross-bridge.

ATPase Activity Measurement. The Ca^{2+}- or K^+(EDTA)-activated ATPase activity was measured by using a modified Fiske and SubbaRow phosphate determination (19). The K^+(EDTA)-ATPase activity was measured in 0.6 M KCl/6 mM EDTA/25 mM Tris·HCl, pH 8, at 25°C. The Ca^{2+}-activated ATPase was measured in 0.6 M KCl/1 mM $CaCl_2$/25 mM Tris·HCl, pH 8, at 30°C. The protein concentration in the assay was 0.02–0.03 mg/ml. The ATPase activities (mean ± SD) of unlabeled S1 were 4.82 ± 0.02 μmol of phosphate per mg of S1 per min for K^+(EDTA)-ATPase and 0.62 ± 0.03 μmol of phosphate per mg of S1 per min for Ca^{2+}-ATPase. These experiments showed that the percent inhibition of the K^+(EDTA)-ATPase was proportional to the dye incorporation, whereas the Ca^{2+}-ATPase was activated by a factor of 2.5, indicating specific modification of SH1.

Tryptic Digestion of S1. Labeled and unlabeled S1 was fragmented with trypsin according to Bálint *et al.* (20, 21). The digestion was carried out in 50 mM Tris·HCl at pH 8 and 25°C with an S1:trypsin ratio of 1:80.

The peptides of the digest were isolated by SDS/PAGE according to Laemmli (22). The acrylamide concentration of the gel was 15%. Gels were analyzed for fluorescence after finishing the electrophoresis and for peptide composition by staining the peptides with Coomassie brilliant blue. Fig. 3

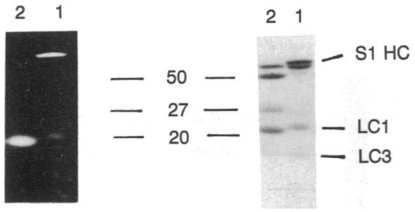

FIG. 3. Localization of IATR in S1 by SDS/PAGE of the tryptic digest of fiber S1. HC, heavy chain; LC, light chain. (*Left*) Gel under UV illumination. Lane 1, S1; lane 2, tryptic digest of S1. (*Right*) Same as in *Left* after Coomassie staining. By using tryptic digestion we split the S1 heavy chain into the three fragments of molecular mass 20, 50, and 27 kDa. The IATR was localized on the 20-kDa fragment containing the fast-reacting thiol SH1.

shows the localization of the IATR on the 20-kDa tryptic fragment of S1.

Quantitative Fluorescence Measurements. The spectroscopic measurements were performed on a SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL) equipped with Glan-Thompson polarizers. The emission was collected at 90° from the excitation beam path. The emission wavelength was selected with a band pass filter transmitting a wavelength band width of 40 nm centered at $\lambda_{em} = 550$ nm. We oriented the fiber so that the fiber axis was perpendicular to the excitation and collected emission beams.

A fluorescence polarization experiment consisted of collecting the fluorescence intensities $F_{\perp,\perp}$ and $F_{\perp,\parallel}$ from the IATR-modified fibers as a function of the excitation wavelength. The first (second) index of F corresponds to the direction of the linear polarization of the excitation (emission) beam. Parallel ($\parallel$) and perpendicular ($\perp$) are directions relative to the fiber axis. These quantities have an identical but arbitrary normalization so that they are combined in the ratio,

$$P_\perp = \frac{F_{\perp,\perp} - F_{\perp,\parallel}}{F_{\perp,\perp} + F_{\perp,\parallel}}. \quad [1]$$

We summarize our data by plotting $P_\perp(\lambda_{ex})$. $P_\perp = 0$ for all λ_{ex} for a random distribution of probes. For an oriented sample, if $P_\perp$ changes sign a probe rotation is unambiguously indicated.

Effect of Nonspecific Probe Emission on $P_\perp$. We include the effect of nonspecific labeling in the muscle fiber on $P_\perp$ by generalizing Eq. 1 to,

$$P_\perp = \frac{F^s_{\perp,\perp} - F^s_{\perp,\parallel} + F^n_{\perp,\perp} - F^n_{\perp,\parallel}}{F^s_{\perp,\perp} + F^s_{\perp,\parallel} + F^n_{\perp,\perp} + F^n_{\perp,\parallel}}, \quad [2]$$

where superscripts s and n refer to specific and nonspecific probes. Eq. 2 can be rearranged into the form,

$$P_\perp = \frac{P^s_\perp + \gamma P^n_\perp}{1 + \gamma}, \quad [3]$$

where

$$\gamma = \frac{F^n_{\perp,\perp} + F^n_{\perp,\parallel}}{F^s_{\perp,\perp} + F^s_{\perp,\parallel}}. \quad [4]$$

From Eq. 3 we can plot $P^s_\perp$ vs. $P^n_\perp$ for observed $P_\perp$ values if we know the value of γ. When $\gamma = 0$ the nonspecific probes contribute nothing to the observed $P_\perp$. In a worst case calculation, we maximize γ, with the side conditions that certain related parameters are equal to their experimentally observed values. In the case of the IATR label on SH1, we require $P_\perp$ and $P_\parallel$ where

$$P_\parallel = \frac{F^s_{\parallel,\parallel} - F^s_{\parallel,\perp} + F^n_{\parallel,\parallel} - F^n_{\parallel,\perp}}{F^s_{\parallel,\parallel} + F^s_{\parallel,\perp} + F^n_{\parallel,\parallel} + F^n_{\parallel,\perp}} \quad [5]$$

to be equal to their observed values while maximizing γ. Our calculations show that $\gamma = 0$ is the only solution consistent with all of the side conditions.

Fiber Stretching Instrument. Fig. 4 shows the instrument used to stretch fibers. The 1.4 × 3.0 cm rectangular stainless steel support fitted inside a standard fluorescence cuvette. The vertical supports were hollow tubes enclosing the thinner stainless steel wire that formed the upper part of the horizontal supports upon which the fibers were mounted. The upper horizontal support was fixed to a micrometer spindle that on turning could accurately change the distance between the lower and upper support in increments of 25 μm. The cap on the cuvette was the static element that held the vertical supports inside the cuvette and relative to which the micrometer spindle moved one end of the fiber. One to 10 fibers were mounted in the instrument at one time. The fibers were illuminated with a focused excitation beam from the fluorescence spectrometer. Only the middle section of the fiber bundle was illuminated.

Fiber Tension Measurements. A protocol similar to that of Goldman *et al.* (14) was applied to the IATR-modified fibers in order to verify that the modified fibers can also maintain negative tension. Bundles of one to 10 modified fibers, prepared identically and of identical size to those used in the fluorescence polarization experiments, were mounted on a tension transducer and bathed in relaxing solution. A 10% stretch was applied and the relaxed tension was recorded after 10 min. This bundle was then thoroughly washed in rigor solution and the steady-state rigor tension was recorded after 5 min. The tension of the rigor fiber bundle was released by allowing the bundle to slacken and the tension dropped to zero, indicating that a negative stress was applied to the cross-bridges by the passive force. After 3 min (the usual time we allowed in a fluorescence polarization measurement) the labeled fiber bundle was returned to relaxing solution and the resting tension was remeasured. We found that the final resting tension was identical to the resting tension induced by the initial 10% relaxed stretch. These results are summarized in Fig. 5 and verify that the probe-modified fibers support

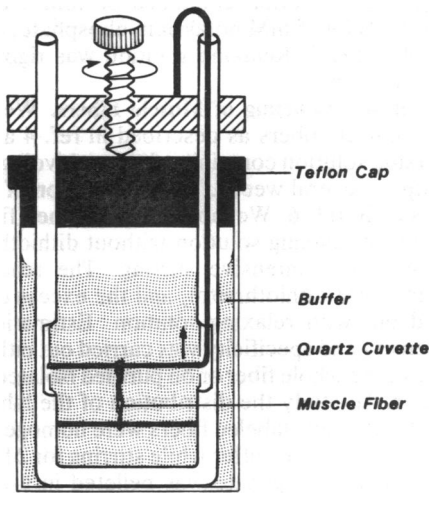

FIG. 4. The fiber stretching instrument consists of a stainless steel rectangle that fits inside a quartz fluorescence cuvette. The vertical supports enclose a thinner wire that forms the upper part of the horizontal supports on which the fiber is mounted. The upper support moves from 0 to 150% of the fiber length in increments of 0.5% by the clockwise rotation of the micrometer mounted on the cap of the fluorescence cuvette; the lower horizontal support is immobile. The moveable support lengthens the fiber against a force supplied by a spring (the spring is not shown in the drawing) so that tension is released by the counterclockwise rotation of the micrometer.

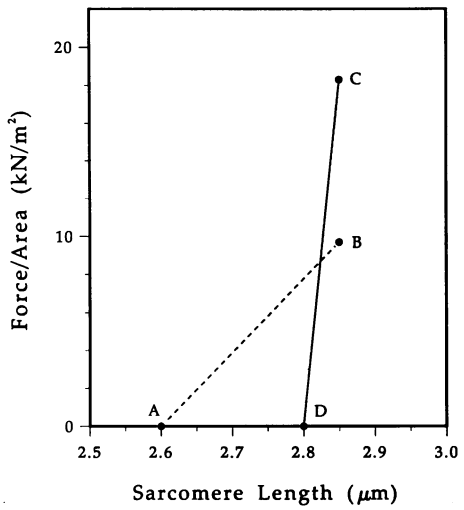

FIG. 5. Fiber tension as a function of length in rigor and relaxation. Relaxed fibers at resting length are stretched 10% of their length. Relaxed tension (---) increases as the fiber is stretched (AB). The stretched relaxed fibers are placed in rigor and tension again increases (BC) without a length change. The rigor tension (—) decreases to zero with a length change of ≈0.05 μm (CD). At point D (the state where we do the fluorescence polarization experiments) there is negative tension on the cross-bridges.

negative forces in a manner identical to that of unmodified fibers.

RESULTS

Shown in Fig. 6 are typical fluorescence polarization spectra measured from IATR-labeled fibers in rigor at resting length, in rigor after the application of negative stress, and in rigor when there was no overlap between the actin and myosin filaments. We plot the polarization ratio $P_\perp(\lambda_{ex})$, defined in Eq. 1, for these cases. The unstressed polarization spectrum is characteristic of IATR probes on SH1 in fibers in the rigor state (corresponding to part a in the experimental protocol of Fig. 1). The negatively stressed cross-bridge polarization spectrum indicates the probe has rotated relative to its orientation on the unstressed cross-bridge (corresponding to part c in Fig. 1). The polarization spectrum from IATR-labeled fibers in the absence of nucleotide at no overlap between the actin and myosin filaments indicates a slight amount of angular order in the probe distribution. No possible linear combination of the no overlap spectrum with the unstressed cross-bridge spectrum will give the negatively stressed cross-bridge spectrum. This indicates that the change in the spectrum due to the application of a negative stress cannot be explained by the introduction of disorder from the small fraction of cross-bridges at no overlap due to the initial relaxed stretch necessary to induce a negative stress.

Also shown in Fig. 6 is the averaged difference spectrum of negatively stressed from unstressed cross-bridges. Error bars indicate standard error of the mean for 13 different fiber preparations. The curve is always positive, indicating that the negative stress always displaced the polarization spectrum to lower values of $P_\perp$. This difference curve used negative stress spectra induced by a 10% stretch in relaxation (corresponding to part b in Fig. 1). Curves like this, resulting from longer relaxed stretches, were higher, indicating a larger angular displacement of the probe when more negative stress was applied. There was no effect of positive stress on rigor cross-bridge orientation as judged by the polarization spectrum (data not shown).

Fibers that were stretched in relaxation, put into rigor, but not released (i.e., negative tension was not allowed to develop on the rigor cross-bridges) have a polarization spectrum identical to that from unstressed rigor (data not shown). This result is expected since the rigor cross-bridges developed normal rigor tension whether or not the fiber had resting tension due to the initial stretch in relaxation.

The effect of negative stress on the polarization spectrum—i.e., the shifting downward of the polarization spectrum—was reversed when negatively stressed rigor fibers were subsequently positively stressed by pulling on the ends of the fiber. Restretching (corresponding to part d in Fig. 1) shifted the polarization spectrum to be approximately on the median between the negatively stressed spectrum and the unstressed spectrum. For instance, we observed $P_\perp(\lambda_{ex} = 500\ nm) = 0.095 \pm 0.01$ from the restretched fibers. This value lies on the mean value of $P_\perp$ from fibers in rigor and negatively stressed fibers in rigor (see Fig. 6). No amount of

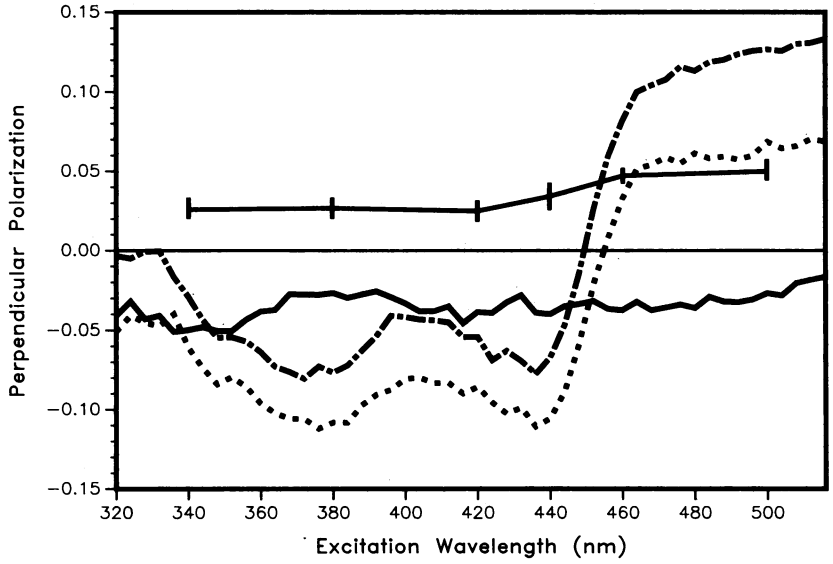

FIG. 6. $P_\perp$ as a function of excitation wavelength for rhodamine-labeled fibers in rigor at resting length (–·–), after the application of negative stress (· · ·), and at no overlap between myosin and actin (—). These data are representative spectra. The vertical error bars and solid line indicate the standard error of the mean (using 13 different fiber preparations) of the difference spectrum between negatively stressed and unstressed rigor cross-bridges as a function of wavelength.

restretching of the negatively stressed fibers could restore the original unstressed polarization spectrum.

In control experiments we determined the effect of the application of positive and negative stress on the rigor cross-bridges on the angular order of the actin filaments. We measured $P_\perp(\lambda_{ex})$ from IATR-labeled myosin subfragment 1 (S1) decorating unlabeled muscle fibers in rigor (data not shown). We found that the application of positive or negative stress on the intrinsic fiber myosin cross-bridge had no effect on the orientation of the decorating IATR-S1. This result shows that the applied stress does not alter actin filament order so that the changes in the polarization spectrum of labeled fibers induced by negative stress are the result of cross-bridge rotation and not of any disordering of the fiber structure.

DISCUSSION

The question of whether cross-bridges can be reoriented by positive or negative stress has been investigated by a spectroscopic probe method. This method incorporated certain advances and precautions. For example, the polarized emission of the probe was studied at various exciting light wavelengths; this is equivalent to repeating the experiment with many probes, each sitting in different attitudes within the cross-bridge. Also, the possibility that the results were confused by the small fraction of cross-bridges at no overlap (due to the initial 10% relaxed stretch) was excluded, as was the possibility that the results were due to propagated disorder of the thin filaments.

As have others before us, we found no evidence that rigor cross-bridges can be rotated by positive stress (11–13, 24). On the other hand, we found clear evidence that cross-bridges can be rotated by negative stress. This result is consistent with the idea that cross-bridges functionally rotate. The asymmetrical results with positive and negative stress are most easily explained by assuming that in the mechanical circuit there is an asymmetrical compliance between the cross-bridge and the filament—i.e., an element that can lengthen with positive tension but is incompressible with negative tension. The effect of negative stress was only partially reversed by the subsequent application of positive stress, suggesting that the negative stress caused an irreversible rotation of a fraction of the labeled cross-bridges. It is possible that the negative stress pushes the fraction of the cross-bridges in a rigor fiber that are not in their minimum free energy state, due to the constraints of the fiber lattice, into a lower free energy state. These cross-bridges cannot be pulled out of this lower free energy state by positive tension. Cross-bridges already at their minimum free energy, however, can be pushed into a higher free energy state by negative stress and can subsequently be pulled back to their minimum free energy by the application of positive stress.

The role of cross-bridge orientation in the mechanism of muscle contraction was obscured by the finding that it was apparently impossible to pull a rigor cross-bridge out of the rigor state orientation by applying a positive tension to a fiber. The direct coupling of fiber length with cross-bridge orientation is a logical result for a simple rotating cross-bridge model. That this coupling was not observed implied, to those who continued to favor the rotating cross-bridge model of contraction, that a decoupling element must exist between the myosin head and the myosin filament backbone. This element is commonly incorporated in the modern models of muscle contraction as a way of storing the free energy of ATP hydrolysis inside the myosin molecule (23).

Our findings indicate that this spring-like decoupling element exists. It appears that this spring is flexible enough that it stretches when the cross-bridge is pulled in rigor, so that no cross-bridge rotation is observed, but is rigid enough (incompressible) to put a strain on the cross-bridge that is detected as a cross-bridge rotation, when a negative stress is applied to the fiber. Once the cross-bridge is strained under negative stress, the spring is stiff enough that we can pull some of the strain off of the cross-bridge when positive tension is subsequently applied. This apparent asymmetry could be due to the cross-bridge being more flexible under negative stress than positive stress or to the spring element being more flexible under positive stress than negative stress, or to both.

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