Transients of Fluorescence Polarization in Skeletal Muscle Fibers Labeled with Rhodamine on the Regulatory Light Chain

Taylor St. Claire Allen,* Cibele Sabido-David,‡ Nicholas Ling,§ Malcolm Irving,‡ and Yale E. Goldman*
*Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104 USA; ‡Molecular Biology and Biophysics Group, Randall Institute, King's College London, United Kingdom; §Department of Biological Sciences, Waikato University, Hamilton, New Zealand

ABSTRACT Structural changes of the myosin heads were correlated with mechanical events in the cross-bridge cycle by measuring fluorescence polarization signals at high time resolution from rhodamine probes bound to myosin regulatory light chains in skeletal muscle fibers. Motions of the cross-bridges were partially synchronized either by applying quick length changes to the fibers during active contractions or by activating the fibers from rigor by photolysis of caged ATP in the presence of Ca^{2+}. With fibers in rigor, the fluorescence polarization values indicate that the probe dipoles are quite well ordered and are directed away from the muscle fiber axis. After photorelease of ATP from caged ATP, changes in polarization signals are consistent with broadening of the distribution of probe orientations. The signal deflections occur when ATP binds to actomyosin or when the cross-bridges detach, but the orientational distribution changes surprisingly little during active force development. In contrast, when staircases of quick releases are applied to labeled fibers during active contractions, the fluorescence polarization signals suggest a concerted rotation of the probes. The results indicate that the light chain region of myosin tilts during the quick release and/or during the tension recovery phase within the next few ms.

INTRODUCTION

The tilting-head model of force generation in muscle postulates that a rotation of the myosin head leads to force generation or, when the external load is low, 5–10 nm of relative sliding of the thick and thin filaments (H. E. Huxley, 1957, 1969; Reedy et al., 1965; A. F. Huxley and Simmons, 1971). Many structural and spectroscopic experiments have shown that myosin is flexible, and therefore the heads can rotate (Cooke, 1986), but none have obtained a direct linkage of myosin rotational motions to the force-generating step or to filament sliding (Cooke et al., 1984; Thomas, 1987).

Fluorescence polarization using extrinsic probes on the myosin head is a technique sensitive only to the angle of the probe molecules relative to the fiber axis. If a probe is attached to part of the myosin that tilts in the power stroke, the angle of the probe molecule and the polarization of its fluorescence are expected to change. In the present experiments we placed rhodamine probes on the regulatory light chain of the myosin head in rabbit *psoas* fibers. To overcome the problem of the multiplicity of states during steady contractions, we introduced sudden perturbations (quick length changes or photolysis of caged ATP), which partly synchronize the motions of the cross-bridges. The fluorescence polarization was measured with sufficient time resolution after these perturbations to detect the expected motions in the time range of several hundred μs up to 100 ms. The results were markedly different for these two perturbations and provide useful implications for the kinetics of the force-generating transition.

MATERIALS AND METHODS

Glycerol-extracted single fibers from rabbit *psoas* muscle were used for the experiments. Dr. J. Kendrick-Jones (MRC, Cambridge) kindly supplied wild-type chicken gizzard myosin regulatory light chains (RLCs) produced by expression of the protein in *E. coli* (Rowe and Kendrick-Jones, 1992). Chicken gizzard myosin regulatory light chain has only one cysteine residue (cys-108) that can be labeled specifically with a fluorescent probe. It also binds tightly and specifically to rabbit myosin subfragment-1 (Trybus and Chatman, 1993). We used iodoacetamidotetramethyl rhodamine (IATR, Molecular Probes (Eugene, OR) lot 10A, probably containing predominantly the 6-isomer; Sabido-David et al., 1994). In the present experiments, ~30% of the RLC was labeled with rhodamine. The labeled RLC was exchanged for the endogenous RLC by a procedure based on that of Moss et al. (1982).

The apparatus for activating the fibers by photolysis of caged ATP was essentially as described in Goldman et al. (1984a). Briefly, a frequency-doubled ruby laser provided 50-ns pulses of 347 nm near-UV light for photolysis of caged ATP. The contents of the trough were saved for later analysis of ATP photoliberation by HPLC. Normal active isometric contractions were also initiated as described in Dantzig and Goldman (1985). During the active contractions, quick length changes were imposed using a moving coil motor kindly provided by Dr. V. Lombardi (University of Florence, Florence, Italy). In both perturbation experiments fiber tension was detected by a silicon semiconductor strain gauge (5 kHz bandwidth, Goldman and Simmons, 1984). Fiber stiffness was monitored at 2 kHz. Solutions and experimental temperatures are listed in the figure legends.

Fluorescence polarization of the probe molecules in the fiber was measured with 500 μs time resolution using a method similar to that described by Tanner et al. (1992). Briefly, collimated 514 nm light from a continuous argon ion laser was passed through a photoelastic modulator which modulated its polarization at 84 kHz and then projected onto the muscle fiber to excite rhodamine fluorescence. Fluorescence emission at $\lambda > 570$ nm was split into components parallel and perpendicular to the fiber axis by a Wollaston prism and collected by two photomultiplier tubes. Fluorescence

Address reprint requests to Dr. Yale E. Goldman, Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6085. Tel.: 215-898-4017; Fax: 215-898-2653; E-mail: goldman@a1.mscf.upenn.edu.

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polarization ratios of the following form were computed offline:

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

where each I is fluorescence intensity with the presubscript indicating polarization of the exciting light relative to the muscle fiber axis and the postsubscript indicating direction of the detector polarization. These polarization ratios provide information on the distribution of the probe absorption dipole orientations relative to the muscle fiber axis (axial angles); if the absorption dipoles are preferentially aligned along the muscle fiber axis, $Q_{\parallel}$ is larger than $Q_{\perp}$; if the absorption dipoles are perpendicular to the muscle fiber axis, $Q_{\perp}$ is the larger ratio. If the probes are isotropically distributed in space, $Q_{\parallel}$ and $Q_{\perp}$ are equal. The difference between $Q_{\parallel}$ and $Q_{\perp}$ is a rough indication of the degree of order in the probe array, but quantitative interpretation requires adopting a specific model of the orientation distribution.

RESULTS

Fig. 1 shows a typical recording of the force, stiffness, and fluorescence polarization ratios during activation by photolysis of caged ATP. The fiber is initially in rigor in the presence of 10 mM caged ATP and ~30 μM free Ca^{2+}. Fiber stiffness is maximal and rigor force is moderate. In rigor, $Q_{\parallel}$ and $Q_{\perp}$ averaged 0.17 ± 0.03 and 0.52 ± 0.01 (mean ± SD; $n = 14$), respectively, indicating that the probe molecules are fairly well ordered and aligned rather perpendicular to the muscle fiber axis. On photoliberation of 2.1 mM ATP by photolysis of caged ATP (arrow), tension decreases indicating cross-bridge detachment, then rises indicating reattachment and force generation. Tension finally plateaus at a value (~200 kN/m^2) representative of the isometric fully active state. Stiffness decreases on photolysis and plateaus at a moderate value suggesting a large proportion of the cross-bridges are attached during the contraction. $Q_{\parallel}$ increases and $Q_{\perp}$ decreases promptly on release of the ATP, but the polarization ratios change little during the subsequent phase of force development. $Q_{\parallel}$ and $Q_{\perp}$ averaged 0.31 ± 0.02 and 0.48 ± 0.01 ($n = 14$), respectively, during the steady contraction initiated by caged ATP photolysis. The shift of the two fluorescence polarization traces toward each other indicates substantial axial rotations of the probes. We interpret the decreased separation between $Q_{\parallel}$ and $Q_{\perp}$ as a disordering of the angular distribution. The transition between the ordered rigor distribution and the disordered active distribution takes place with ~5 ms half-time, either before or concomitant with cross-bridge detachment. Surprisingly, there is little evidence, if any, for a concerted angle change during the subsequent force development phase.

Fig. 2 shows the tension, length change, and polarization ratio signals when a series of quick releases (each ~5 nm/half-sarcomere) were applied to an actively contracting muscle fiber at 50-ms intervals. In this case, the fiber was activated by transfer to a solution with 5 mM MgATP, ~30 μM free Ca^{2+}, and no caged ATP. The initial values of $Q_{\parallel}$ and $Q_{\perp}$ (average 0.39 ± 0.01 and 0.451 ± 0.002, respectively; $n = 8$) are similar to the values present at the end of Fig. 1. The traces in Fig. 2 are the average of eight trials performed in a fiber to improve the signal-to-noise ratio, and so the Q traces are smoother than in Fig. 1.

Each length step, completed in less than 200 μs, initiates a decrease of tension simultaneous with the length change, partial recovery within the next 1–2 ms, and then the beginning of further recovery. Even though the full recovery does not take place within the 50-ms interval between steps, the quick (1–2 ms) recovery is largely reprimed. Thus the initial phases of each repeated transient are similar from one step to the next as previously reported for frog muscle (Lombardi et al., 1992). At each quick release, the $Q_{\perp}$ signal increases abruptly and then decays back to the isometric value

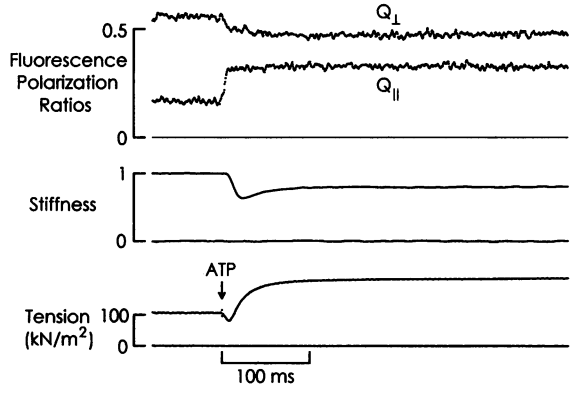

FIGURE 1 Mechanical and orientational signals during activation of a fiber by photolysis of caged ATP. The fiber was immersed in a solution containing (concentrations in mM) TES buffer, 100; CaEGTA, 20; HDTA, 16.6; $MgCl_2$, 3.3; reduced glutathione, 10; caged ATP, 10; free Mg^{2+}, 1; free Ca^{2+} 0.03; ionic strength, 200. At the time of the arrow 2.1 mM ATP was released from caged ATP by a 347 nm UV light pulse from a frequency-doubled ruby laser. Optical signals ($Q_{\parallel}$ and $Q_{\perp}$) are explained in the text. The mechanical signals were recorded as described by Goldman et al. (1984a, b). Fiber dimensions: length 2.76 mm, sarcomere length 2.34 μm, cross-sectional area 6031 μm^2, $T = 21$°C.

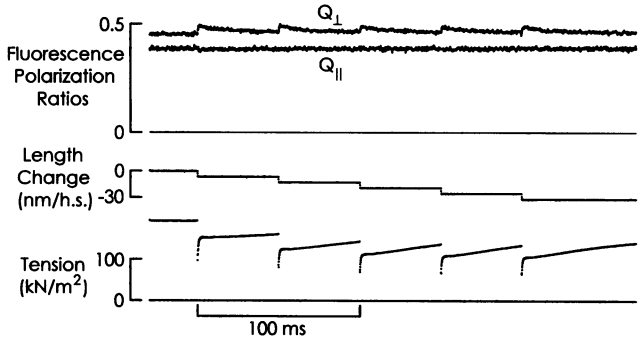

FIGURE 2 Mechanical and optical signals during a staircase of applied quick releases. The length change in each step, uncorrected for end compliance, would correspond to 6 nm/half-sarcomere. In other experiments, this step amplitude caused 4.8–5.0 nm shortening/half-sarcomere as measured by white light diffraction (Goldman, 1987). The fiber was immersed in a solution containing (concentrations in mM) TES buffer, 100; CaEGTA, 25; $MgCl_2$, 6.9; Na_2ATP, 5.62; reduced glutathione, 10; creatine phosphate, 22.4; creatine phosphokinase, 250 units/ml; free Mg^{2+}, 1; free Ca^{2+} 0.03; ionic strength, 200. Signals were recorded as in Fig. 1 except that the traces are averages of staircases applied in eight successive contractions to improve signal-to-noise ratio. Fiber dimensions: length 2.47 mm, sarcomere length 2.43 μm, cross-sectional area 8,013 μm^2, $T = 11$°C.

during the next 50 ms. $Q_{\parallel}$ decreases slightly at each step. This small decrease is not clear at the scale plotted in Fig. 2.

Since the signals from each step in Fig. 2 seem similar, the individual steps in this recording and those from several equivalent contractions of the fiber were averaged together and then plotted on a faster time base in Fig. 3. The increase of the $Q_{\perp}$ signal seems to accompany the quick recovery phase of the tension transient, but at the present 500-μs time resolution, we cannot distinguish events simultaneous with the length change from those immediately following it. The small decrease in $Q_{\parallel}$ does seem to be faster than quick tension recovery. Based on recent higher time-resolution experiments (Allen et al., 1994), and using pure isomers of the IATR (Corrie and Craik, 1994), the transient increase of $Q_{\perp}$ occurring with each length change in Figs. 2 and 3 has been found to take place partly during the length change itself and partly later, during the quick recovery. Modeling the orientation distribution as a single homogeneous population with static and dynamic disorder, the deflection of the polarization ratio signals corresponds to a 2–3° rotation. The angle change is larger if not all sites are involved. Although the changes in probe orientation detected in this experiment seem relatively small, the data provide strong evidence for a concerted rotational motion of the RLC site during the force changes involved with a quick release and the quick tension recovery.

DISCUSSION

The experiments reported here are among the first to directly associate cross-bridge angle changes with force development in muscle fibers. During and after quick releases in active contractions, we obtained small changes of fluorescence polarization with fibers labeled on the RLCs with rhodamine probes. The deflections of $Q_{\parallel}$ and $Q_{\perp}$ are toward their rigor values, perhaps indicating changes toward the structural configuration at the end of the power stroke.

In experiments analogous to Fig. 2 of this work, Irving et al. (1992) found that the 14.5 nm meridional x-ray reflection from frog single, intact muscle fibers, indicative of the axial mass distribution of myosin heads, decreases during the quick recovery after length changes. The decrease in intensity of the 14.3 nm reflection can be interpreted as an angle change of the myosin heads, but changes in shape or longitudinal order between the heads could also decrease the intensity. As mentioned, the fluorescence polarization technique used here is sensitive only to the axial angle.

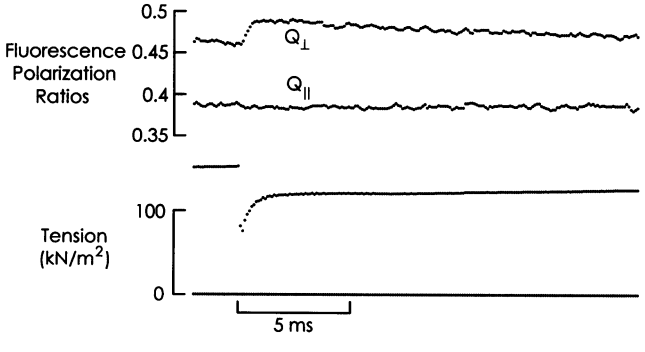

FIGURE 3 Tension and optical signals averaged from staircase length steps. The signals from a series of 40 length steps during eight activations were averaged together. Conditions and fiber dimensions as in Fig. 2.

If a rotational motion occurs during the force-generating step of the cross-bridge cycle, why have so many studies failed to detect it clearly? Several factors might have hindered detection of an angle change. 1) The ATPase activity of actomyosin during a normal muscle contraction distributes the heads among all of the biochemical intermediates on the pathway of the enzymatic reaction, and the angular distribution should reflect the inhomogeneous population. In fact many experimental techniques indicate disorder among the heads during contraction (H. E. Huxley et al., 1982; Cooke et al., 1982; Tsukita and Yano, 1985; Irving, 1993). 2) The size of the expected molecular motion (5–10 nm) is near the present limit of resolution for quick-freezing, freeze-substitution electron microscopy (Hirose et al., 1993). 3) The angle change might actually be very small. 4) Spectroscopic probes might be situated on myosin at a position or angle unfavorable for detecting axial motions. 5) Although detailed mechanical studies on intact muscle fibers suggest an elementary mechanical stroke in the 5–10 nm range consistent with the 16–20 nm size of the myosin head, some experiments imply multiple power strokes per enzymatic cycle (Lombardi et al., 1992; Irving et al., 1992) or a much longer power stroke (Yanagida et al., 1985; Harada et al., 1990).

The changes in polarization signals of Figs. 2 and 3 are much smaller than would be expected if a large proportion of cross-bridges rotate on the order of 45° as suggested in many models of the contraction mechanism. We cannot rule out that our probes are situated at an unfavorable position or angle. Another possible explanation for the small size of the signal deflections is that only a small proportion of cross-bridges are producing force at any instant during contraction. There might be substantial cross-bridge populations detached or in disordered, attached, preforce states. Then, even if the force-generating cross-bridges have a markedly different orientation or more order than the average attachment, their contribution to the observed signal would be small. This interpretation may be difficult to reconcile with the relatively high stiffness during contraction (Fig. 1). The high cross-bridge force required to explain total force if only a small proportion are active would also lead to difficulties in modeling the kinetics of the quick recovery following length steps (V. Lombardi, personal communication).

When the muscle fibers were activated by caged ATP photolysis, the main angle changes preceded force development. An apparent disordering occurred either with ATP binding or with cross-bridge detachment. This disordering is expected since our own fluorescence polarization signals from relaxed muscle fibers are consistent with a wide angular distribution and other methods detect mobility on the μs time scale in detached cross-bridges (Thomas, 1987).

The Q traces in Fig. 1 show very slight changes during active force development, but this slow component was not present in all experiments and is opposite to the direction of the deflections observed in Figs. 2 and 3 during the quick force redevelopment phase following the length steps. Why are the changes of polarization ratios during force development following the quick releases so different from those in the caged ATP experiments? The elementary force-generating transition may occur very quickly relative to the onset of force in caged ATP experiments such as in Fig. 1. This idea is consistent with a force-generating transition concomitant with quick recovery after a length step. The length step and caged ATP protocols would then reveal different transitions. What, then, limits the onset of force development following caged ATP photolysis? ATP hydrolysis and P_i release are kinetically important. Another factor is filament sliding, since sarcomeres are not clamped isometric. Cross-bridges thus may execute several force-generating impulses during tension development after ATP release. If so, the rate of repeated power strokes would be several times higher than the rate of ATP splitting (Ferenczi et al., 1984). This hypothesis would imply loose coupling between the biochemical and mechanical cycles, as suggested from other considerations (Yanagida et al., 1985; Higuchi and Goldman, 1991; Lombardi et al., 1992.).

In summary, we observed angle changes in a fluorescent probe placed on the RLCs of myosin in rabbit *psoas* fibers either when an active muscle was quickly released by a few nm per half-sarcomere or when a rigor muscle was suddenly activated by photorelease of ATP from caged ATP. The two perturbations caused very different changes in the probe orientation distributions. In the quick length change experiment, the deflections in fluorescence polarization signal correlated with the length change and with subsequent force development. This result provides strong evidence for cross-bridge tilting motions associated with these events.

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