Studies on Conformation of F-Actin in Muscle Fibers in the Relaxed State, Rigor, and during Contraction Using Fluorescent Phalloidin

EWA PROCHNIEWICZ-NAKAYAMA, TOSHIO YANAGIDA, and FUMIO OOSAWA
Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Osaka, Japan

ABSTRACT F-actin in a glycerinated muscle fiber was specifically labeled with fluorescent phalloidin-(fluorescein isothiocyanate) FITC complex at 1:1 molar ratio. Binding of phalloidin-FITC to F-actin affected neither contraction of the fiber nor its regulation by Ca^{2+}. Comparison of polarized fluorescence from phalloidin-FITC bound to F-actin in the relaxed state, rigor, and during isometric contraction of the fiber revealed that the changes in polarization accompanying activation are quantitatively as well as qualitatively different from those accompanying transition of the fiber from the relaxed state to rigor. The extent of the changes of polarized fluorescence during isometric contraction increased with decreasing ionic strength, in parallel with increase in isometric tension. On the other hand, polarized fluorescence was not affected by addition of ADP or by stretching of the fiber in rigor solution. It is concluded from these observations that conformational changes in F-actin are involved in the process of active tension development.

Early studies on the properties of skeletal muscle actin suggested that some transformations of the polymer structure upon interaction with myosin are involved in the mechanism of contraction (1). Later findings that F-actin is a flexible polymer and that its flexibility increases upon binding of myosin heads in vitro as well as in vivo (2–10) and that immobilization of thin filaments in muscle ghost fibers by cross-linking with glutaraldehyde inhibits development of isometric tension (11) supported such a possibility. In this paper we compare directly the conformation of F-actin in a glycerinated muscle fiber in rigor, the relaxed state, and during isometric contraction. As a probe for monitoring conformational changes in F-actin, a phalloidin-fluorescein complex (phalloidin-FITC[1]), which is a fluorescent derivative of mushroom toxin phalloidin, was used.

FITC-labeled phalloidin has been already successfully used for visualization of F-actin bundles in nonmuscle cells (12). We have found that phalloidin-FITC can be bound to F-actin in a glycerinated muscle fiber without affecting either tension or its regulation by Ca^{2+}. Since, in skeletal muscle, F-actin filaments are aligned parallel to the axis of the muscle fiber, we could measure the polarized fluorescence from the bound fluorophore using light polarized either perpendicular or parallel to the axis of the fiber. The different values of polarization obtained in the rigor, relaxed, and active states of the fiber suggest that specific conformational changes in F-actin take place during development of isometric tension. A preliminary report of a portion of these results has appeared (13).

MATERIALS AND METHODS

Materials: Glycerinated fibers from rabbit psoas muscle with a sarcomere length from 2.0 to 3.8 μm were prepared according to the method described previously (6). A single glycerinated fiber (diameter, 50–60 μm, effective length, 1 mm) was washed with rigor solution (100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$) and labeled by incubation for 20 min at 6°C in rigor solution containing, in addition, 5 μM phalloidin-FITC. Unbound dye was removed by washing the fiber with rigor solution. The concentration of incorporated phalloidin-FITC was calculated from the fluorescence intensity of the labeled fiber according to the formula $(B) = (a \times (c))/(b \times 2.2)$, where (B) is the concentration of bound dye, a is the fluorescence intensity from the fiber, b is the fluorescence intensity from the known concentration (c) of the free dye, and 2.2 is a correction factor for the difference between fluorescence intensities of phalloidin-FITC before and after binding to F-actin. The correction factor was calculated from control experiments, which show that fluorescence intensity from 4 or 8 μM dye bound to 8 μM F-actin-tropomyosin-troponin complex (515–545 nm with excitation at 470 nm) is ~2.2 times higher than that from the free dye. The fluorescence intensity from the unbound phalloidin-FITC in rigor solution was negligibly small in comparison with that from the dye bound to the fiber.

Ghost fibers were obtained after removal of myosin and regulatory proteins

[1] *Abbreviation used in this paper:* FITC, fluorescein isothiocyanate.

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from glycerinated muscle fibers by 1-h incubation at 0°C in a Hasselbach-Schneider solution: 0.6 M KCl, 4 mM $MgCl_2$, 4 mM ATP, 2 mM EGTA, and 0.1 M phosphate buffer pH 6.4, containing 1% Triton X-100. To inhibit adenylate kinase activity in the experiments with ADP, 100 μM P1, P5 deadenosine pentaphosphate was added. Ca-EGTA buffer was prepared according to the method described by Ogawa (14), assuming an association constant of $10^{6.68}$ for the Ca^{2+} EGTA complex. Actin, tropomyosin, and troponin from rabbit skeletal muscle were isolated and purified as described previously (6). Myosin was prepared according to the method of Perry (15), and heavy meromyosin was obtained by α-chymotrypsin digestion of myosin (16). Protein concentrations were determined by the biuret method (17). ADP and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Phalloidin-FITC, synthetized according to the method described by Wulf et al. (12), was a gift from Dr. Th. Wieland, Max-Planck-Institute for Medical Research (Heidelberg, Federal Republic of Germany).

Methods: Tryptophan fluorescence from the fiber was measured with a microspectrophotometer with excitation at 297.5 nm, and the emitted light was collected at wave lengths >330 nm (6).

Polarized fluorescence from phalloidin-FITC bound to the F-actin in a single glycernated muscle fiber was measured with a microspectrophotometer according to the method previously described (6). Phalloidin-FITC was excited at 470 nm (±0.2 nm) and the emitted light passing through a band pass filter (525–545-nm FITC filter, Nikon Co., Japan) was collected. The cross-section of the beam was 80 × 100 μm^2. The intensities of the four components of polarized fluorescence: ${}_{\parallel}I_{\parallel}$, ${}_{\parallel}I_{\perp}$, ${}_{\perp}I_{\perp}$, ${}_{\perp}I_{\parallel}$, were measured by this apparatus; subscripts on the left side indicate the direction of the incident light, subscripts on the right side indicate the direction of the emitted light relative to the fiber axis. The subscripts $\parallel$ and $\perp$ denote directions parallel and perpendicular, respectively, to the fiber axis. The degrees of fluorescence polarization, $p_{\parallel}$ and $p_{\perp}$, were defined as:

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

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

Since fluorescence intensities from phalloidin-FITC were very strong to prevent photobleaching effects, the slit width of the monochromator was <0.2 mm (300 W-Xenon lamp). The diameter of the fiber was calculated from the birefringence of the fiber, as previously described (18). Tension of muscle fiber was measured with a tension detector made with a semiconductor element (AE 801; Aksjeseiskapet·Micro Electronikk Co., Norway; resonance frequency, 7 kHz). Sarcomere length was determined by a diffraction method, using an Ne-He laser.

To examine the effect of stretching of the fiber on polarized fluorescence from phalloidin-FITC bound to F-actin, a single fiber was mounted to a stainless-steel needle connected on one end to an electromagnetic coil of a loud speaker (0.63 W; Onkyo Co., Japan) driven by an electronic stimulator (MSE 3R; Nihon Koden Co., Japan). The other end of the fiber was fixed with a tape and colloidion. A stepwise length change of 1% was applied by the electromagnetic coil, and fluorescence polarization and the tension of the fiber were measured. The changes in polarized fluorescence and in tension were recorded with a signal processor (7T07A; Sankei Sokki Co., Japan).

RESULTS

Binding of Phalloidin-FITC to a Single Glycerinated Muscle Fiber

Fluorescence intensity from phalloidin-FITC incorporated into a single glycerinated muscle fiber was not affected by extraction of myosin and regulatory proteins. On the other hand, fluorescent staining with the dye could not be obtained after removal of F-actin from an unlabeled myosin-free ghost fiber by 0.6 M KI. These results prove specificity of binding of phalloidin-FITC to F-actin in muscle fiber.

Concentration of phalloidin-FITC bound to F-actin was calculated from fluorescence intensity of the bound dye, as described in Materials and Methods. Maximum amount, ~670 μM, was obtained when the fiber was incubated for 20 min at 6°C in rigor solution containing 5 μM phalloidin-FITC. Assuming a concentration of actin in the fiber of ~600 μM (19), this corresponds to ~1 mol of the toxin per 1 mol of actin.

It has been well established that phalloidin stabilizes F-actin against depolymerization by KI (20). The fluorescent derivative, phalloidin-FITC, has the same property: while depolymerization of F-actin in an unlabeled ghost fiber results in a decrease in fluorescence intensity from tryptophan residues in the fiber, no change was found after addition of KI to a phalloidin-FITC-labeled ghost fiber (Fig. 1).

Effect of Binding of Phalloidin to F-Actin on Isometric Tension of a Single Glycerinated Muscle Fiber

The experiments in vitro have shown that stoichiometric binding of phalloidin to F-actin in solution did not affect superprecipitation of actomyosin complex, activation of actomyosin ATPase, and Ca-sensitivity of the ATPase in the presence of regulatory proteins (21). According to the data shown in Fig. 2, interaction of actin with myosin in the muscle fiber is not affected by phalloidin or phalloidin-FITC. The time course of tension development, the effect of ionic strength on isometric tension, and Ca-regulation of the tension were the same before and after treatment of the fiber with the toxin.

Polarized Fluorescence from Phalloidin-FITC Bound to F-Actin in a Single Glycerinated Muscle Fiber

POLARIZED FLUORESCENCE IN RIGOR, RELAXING, AND ACTIVATING SOLUTIONS: The effect of rigor, relaxing, and activating solutions on polarized fluorescence from phalloidin-FITC bound to F-actin is shown in Fig. 3. The sarcomere length of the fibers was 2.2 μm, which assures almost full overlap between thin and thick filaments.

When the fiber was transferred from rigor to relaxing solution, the value of $p_{\parallel}$ decreased from 0.410 ± 0.0015 to 0.398 ± 0.001 and that of $p_{\perp}$ increased from 0.062 ± 0.002 to 0.079 ± 0.003. This change of polarized fluorescence was independent of the KCl concentration in the range from 0 to 100 mM (Fig. 3).

The fiber was activated by transfer from relaxing to activating solutions containing 100 mM, 50 mM, and 0 mM

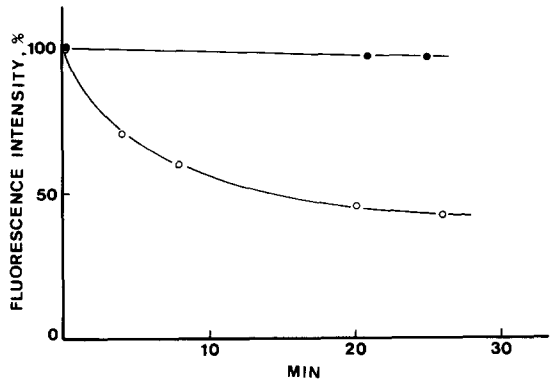

FIGURE 1 Stability of phalloidin-FITC-labeled F-actin in a single glycerinated muscle fiber against depolymerization by KI. A single glycerinated fiber labeled (●) and unlabeled (○) with phalloidin-FITC was treated with a Hasselbach-Schneider solution for 1 h at 0°C to remove myosin and regulatory proteins; at zero time, the ghost fibers were immersed in rigor solution: 100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$ containing 0.6 M KI, and the time course of the changes of tryptophan fluorescence intensity was measured at 23°C.

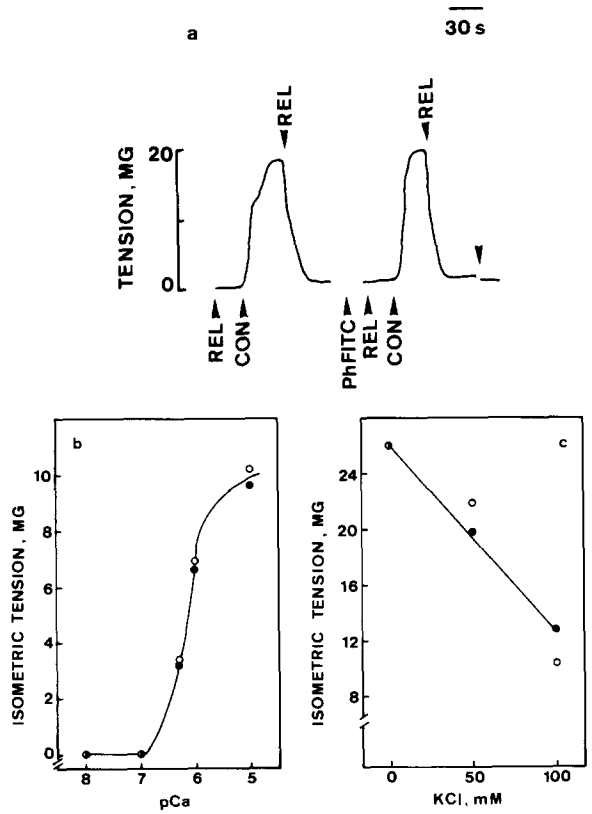

FIGURE 2 Isometric tension of a single glycerinated muscle fiber before and after treatment with phalloidin. (*a*) The time course of tension development. The arrows labeled *Rel* and *Con* indicate the addition of the following solutions to the fiber: *Rel*, relaxing solution: 100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA; *Con*, activating solution: 100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM $CaCl_2$. The arrow labeled *PhFITC* indicates that the fiber was incubated for 20 min at 6°C with 5 μM phalloidin-FITC in rigor solution. (*b*) The effect of Ca^{2+} concentration on isometric tension in activating solution containing 100 mM KCl, 10 mM phosphate buffer pH 7.50, 5 mM $MgCl_2$, 5 mM ATP, and Ca^{2+} concentrations varied as indicated on the abscissa. (*c*) The effect of ionic strength on isometric tension in activating solution containing 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM $CaCl_2$, and KCl concentrations varied as indicated on the abscissa. O, the fiber before treatment with phalloidin; ●, the same fiber after 20-min incubation at 6°C with 5 μM phalloidin in rigor solution. 6°C.

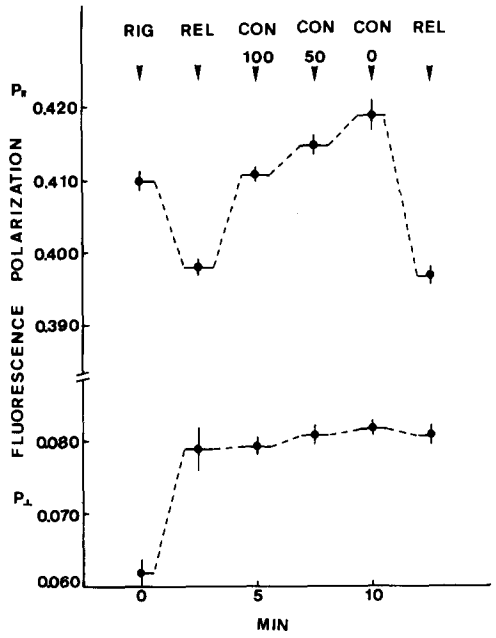

FIGURE 3 Fluorescence polarization from phalloidin-FITC bound to F-actin in a single glycerinated muscle fiber. At the times indicated by the arrowheads, each bathing solution was replaced by a new one, and, after equilibration of the fiber in the new bathing solution for 2 min, polarized fluorescence was measured. *Rig*, rigor solution; *Rel*, relaxing solution: 100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA. *Con*, activating solutions: 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM $CaCl_2$, and KCl concentrations 0, 50, or 100 mM, as indicated by the numbers on the figure. Each point indicates the average value and the vertical bars represent the standard error for eight measurements in which different fibers were used. 6°C.

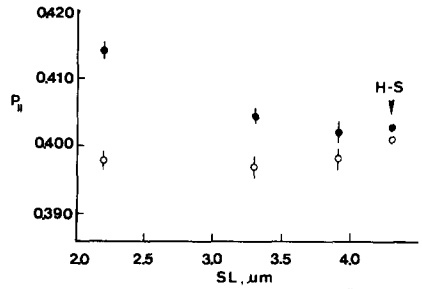

FIGURE 4 The effect of sarcomere length on fluorescence polarization from phalloidin-FITC bound to F-actin in a glycerinated muscle fiber. ●, fluorescence polarization in activating solution: 50 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM $CaCl_2$; O, fluorescence polarization in relaxing solution: 100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA. The arrow labeled *H-S* indicates fluorescence polarization measured after removal of myosin and regulatory proteins from the fiber by 1-h incubation at 0°C in a Hasselbach-Schneider solution. 6°C.

KCl. Such a decrease in ionic strength permitted measurement of polarized fluorescence at increasing values of isometric tension (Fig. 2). During activation $p_\perp$ remained almost constant, but $p_\parallel$ increased and exceeded the value obtained in rigor solution. Furthermore, $p_\parallel$ in activating solution increased with decrease in ionic strength: for example, at KCl concentrations of 100 mM and 0 mM, the values of $p_\parallel$ were 0.411 ± 0.001 and 0.419 ± 0.001, respectively (Fig. 3). Addition of ADP instead of ATP to rigor solution affected neither $p_\parallel$ nor $p_\perp$ values. When myosin and regulatory proteins were completely removed from the fiber by a Hasselbach-Schneider solution, polarized fluorescence from phalloidin-FITC bound to F-actin in the resultant ghost fiber was independent of the kind of bathing solution (Fig. 4).

POLARIZED FLUORESCENCE FROM THE FIBERS WITH VARIOUS SARCOMERE LENGTHS: The data in Fig. 4 show polarized fluorescence from phalloidin-FITC bound to F-actin in muscle fibers with various sarcomere lengths. In the relaxing solution, increase in the sarcomere length from 2.0 to 3.8 μm, where already no overlap between thin and thick filaments is expected, did not affect polarized fluorescence. On the other hand, when the fiber was transferred to activating solution, polarized fluorescence decreased with an increase in sarcomere length and gradually approached values obtained in relaxing solution. Polarized fluorescence measured during contraction was very stable and changed reversibly with the subsequent cycles of contraction-relaxation (Fig. 5).

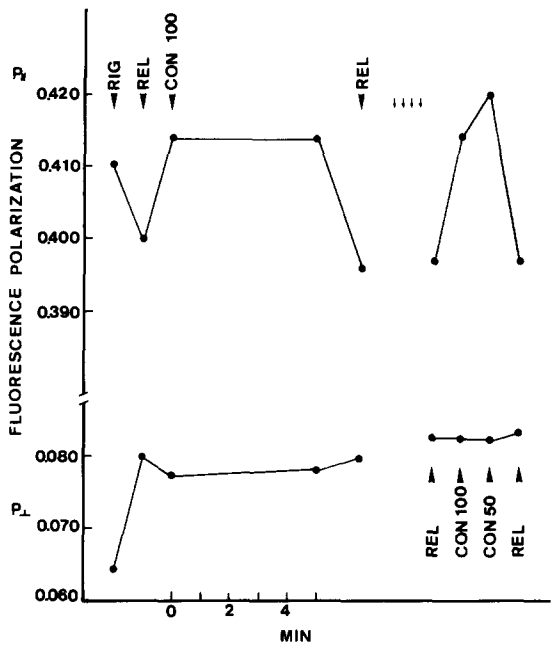

FIGURE 5 Stability of fluorescence polarization from phalloidin-FITC bound to F-actin during isometric contraction. The arrowheads labeled *Rig*, *Rel*, *Con* 100, and *Con 50* indicate the times when the fiber was immersed in the following solutions: *Rig*, rigor solution; *Rel*, relaxing solution: 100 mM KCl, 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA; *Con 100*, *Con 50* activating solutions containing 10 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM $CaCl_2$, and 100 or 50 mM KCl, respectively. Each arrow without the label indicates one cycle of contraction-relaxation of the fiber: Con 100 → Con 50 → Rel. 6°C.

POLARIZED FLUORESCENCE DURING PASSIVE STRETCHING OF THE FIBER: When a single glycerinated fiber was stretched in rigor solution at 6°C, a stepwise length change of 1% resulted in a tension of 1.5 kg/cm^2, which is of the same order as the tension developed by the fiber in activating solution. However, in the present case, no change in polarized fluorescence from phalloidin-FITC bound to F-actin was found.

ESTIMATION OF THE EFFECTS OF NOISE LEVEL, AND LIGHT SCATTERING AND BIREFRINGENCE ON POLARIZED FLUORESCENCE: Noise level was <0.3% of the amplitude of the signal at a time constant = 2 s; therefore its effect on the measured intensities of the four components of polarized fluorescence was considered to be negligibly small.

The extent of light scattering and birefringence was estimated by comparison of the polarization when the incident beam passed through a solution and through an unlabeled fiber. Transfer of such a fiber from relaxing to activating solution changed the value of $p_\parallel$ by ~0.2%, and transfer from relaxing to rigor solution, by ~0.7%; this is about one-tenth of the changes from phalloidin-FITC labeled fiber (the latter were of the order of 3–5%), when measured under the same conditions. In the case of $p_\perp$, the change accompanying transfer of an unlabeled fiber from relaxing to rigor solution was ~7%, which is also considerably less than found under the same conditions ~22% change in $p_\perp$ from phalloidin-FITC-labeled fiber. When an unlabeled fiber was transferred from relaxing to activating solution, $p_\perp$ changed by ~2% and, under these conditions, polarized fluorescence from the labeled fiber did not show essential changes.

Total Fluorescence Intensity from Phalloidin-FITC Bound to F-Actin in Solution in the Presence of Myosin and Regulatory Proteins

Fluorescence intensity from phalloidin-FITC bound to F-actin was measured in rigor, relaxing, and activating solutions after addition of 8 μM heavy meromyosin to 8 μM F-actin-tropomyosin-troponin complex preincubated with 4 or 8 μM phalloidin-FITC. In all these cases, the same values of fluorescence intensities were obtained.

DISCUSSION

Our results show that binding of phalloidin-FITC to F-actin in a glycerinated muscle fiber does not affect isometric tension and Ca-regulation of the tension. On the other hand, in the case of nonmuscle actins, binding of phalloidin has diverse effects: depending on the cell, contractile processes are either not affected (22, 23, 24) or inhibited (25–28). It has been postulated that the inhibitory effect of phalloidin is limited to those cells in which G-F transformation of actin is involved in the mechanism of movement generation. Therefore, permanent organization of muscle actin into ordered array of thin filaments is one explanation of our results. Additionally, the dynamic properties of already polymerized actin, which seem to be very important for contractility (29), are not affected by phalloidin. The shape and bending motion of F-actin in solution, associated with small rotations and/or distortions of the monomers within the filament (30), has the same features in the absence and in the presence of phalloidin (31) or its fluorescent derivatives (32).

The changes in polarization from phalloidin-FITC bound to F-actin, when a fiber is transferred from relaxing to rigor solution, indicate conformational changes in F-actin. Since in the relaxed state myosin is dissociated and in the rigor state almost all myosin heads are associated with actin (33, 34), these conformational changes are possibly induced by formation of rigor bonds. Such a conclusion is consistent with other results (6). Changes in polarization were also observed during activation of the fiber, when only part of myosin (20–40%) interacts with actin (35, 36). However, these changes were quantitatively as well as qualitatively different from those accompanying formation of rigor bonds: $p_\perp$ remained almost constant and $p_\parallel$ increased, surpassing the values obtained in the rigor state. Therefore, conformation of actin during contraction seems to be different than in the relaxed or in rigor state. The extent of changes during activation increased with the extent of isometric tension, indicating that they are induced by specific interactions between actin and myosin, which are involved in the mechanism of tension generation. Such a possibility is further supported by the observations that (*a*) neither addition of ADP nor passive stretching of the fiber affects polarized fluorescence and (*b*) at zero overlap between thin and thick filaments and in myosin-free ghost fibers the values of polarized fluorescence were independent of the kind of bathing solution.

Measurement of polarized fluorescence permits determination of conformation of actin in terms of the angles of absorption and emission dipoles of the fluorophores relative to the F-actin axis (6). The values of polarized fluorescence from phalloidin-FITC bound to F-actin were for example in rigor solution: $p_\parallel = 0.410$, $p_\perp = -0.062$. If the fluorophores are fixed with a helical array, the values should be $p_\parallel = 0.410$,

$p_\perp = -0.372$ (6, 37). Thus, our data show considerable deviation from those theoretically predicted for an ordered array, and quantitative interpretation requires theoretical simulation of disorder. Assuming a Gaussian function for an angular distribution of the dipoles (6), the angles of absorption ϕ_A and emission ϕ_E dipoles at the center of the angular distribution and the width at half maximum of the Gaussian distribution $\Delta\theta$, calculated from the values of polarized fluorescence, are in rigor solution, $\phi_A = 36.5°$, $\phi_E = 38.0°$, and $\Delta\theta = 37.0°$, and in relaxing solution, $\phi_A = 38.5°$, $\phi_E = 39.0°$, and $\Delta\theta = 37.0°$. A few percent change, accompanying activation of the fiber, corresponds to the change of ϕ_E by 2°. However, the calculated values have some ambiguity, because the results of calculation depend on the assumptions of the model: for example, the distribution of the dipoles may be different from the Gaussian one. Deviation of our data from those predicted for a single helix cannot be caused by a nonspecific binding, because phalloidin-FITC binds only to F-actin. Data, obtained in a solution system, indicate that the bound phalloidin-FITC is not directly influenced by myosin or regulatory proteins. Furthermore, the discrepancy is too large to be explained by thermal fluctuations of the thin-filament axis from the fiber axis (6, 7). The most plausible explanation seems to be thermal fluctuation of FITC, which is not directly attached to actin, but through the chain of 1-aminopropane-2,3-dithiol and phalloidin (12). If this is the case, the calculated change of ϕ_E by only a few degrees seems to underestimate the real change in the orientation of actin monomer.

The probe, ϵ-ADP, which is a fluorescent derivative of ADP, can be rigidly bound to actin (38). When ϵ-ADP was incorporated into F-actin in a myosin-free ghost fiber, the obtained values of polarization were very close to those expected for a single helix (6). Preliminary data have shown that when the ghost fiber was irrigated with myosin, the difference in polarized fluorescence from ϵ-ADP bound to F-actin in the relaxed state and during tension development corresponds to the rotation of the fluorophore by $\sim 10°$ (39).

In most models of muscle contraction, only conformational changes of myosin are considered as essential for force generation, and the role of actin is reduced to passive supporting of the movement of myosin heads. Our data indicate that conformational differences in actin can be also involved in the mechanism of contraction.

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