Orientation of Actin Filaments During Motion in In Vitro Motility Assay

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ABSTRACT Rhodamine-phalloidin was added to F-actin, and the orientation of transition dipoles of the dye was measured in single actin filaments by polarization of fluorescence. Rhodamine-phalloidin was well immobilized on the surface of actin, indicating that changes in orientation of the dye reported changes in orientation of actin monomers. In stationary filaments the dipoles were inclined at 49.3° with respect to the filament axis. The disorganization of dipoles in stationary filaments was insignificant. When the filaments were made to translate, the average orientation of the dye did not change, but disorganization slightly increased. Disorganization increased significantly when filaments were free in solution. We concluded that, within the accuracy of our measurements (≈18%), actin monomers did not undergo major reorientations during motion, but that binding of myosin heads deformed the structure of filaments.

INTRODUCTION

The force-generating power stroke in muscle is thought to be caused by change in orientation of myosin head (subfragment-1 or S1) relative to the axis of thin filament (Huxley, 1969). The molecular impeller that causes this change resides at the interface between actin and S1 (Botts et al., 1973; Nihei et al., 1974), but it is not clear whether only the myosin head or both S1 and actin change orientation during contraction. Most studies assumed that myosin was an active entity and that actin served merely as a passive "rail" on which myosin moved (e.g., Borejdo et al., 1979; Stein et al., 1990). However, changes in attitude of S1 can also result from the changes in flexibility of F-actin or from the changes in the series elasticity that may reside in an actin protomer (Huxley and Simmons, 1971). In fact, a significant amount of information suggests that actin plays an active role in contraction. Oosawa et al. (1973) showed by quasi- elastic-light-scattering and by birefringence that the thin filaments undergo increase in the internal motion during ATPase activity. Prochniewicz-Nakayama et al. (1983) showed that during contraction of striated muscle actin monomers undergo small but significant change in orientation and concluded that conformational changes in F-actin were involved in active tension development. Yanagida and Oosawa (1978) and Yanagida (1981) showed that the transition dipole of ϵ-ADP incorporated into thin filaments rotated when heavy meromyosin (HMM) was added and when muscle underwent transition from relaxation to tension development. Recently, Schutt and Lindberg (1992) suggested a specific model in which actin plays an active role in contraction of muscle.

A related question—whether binding of myosin heads to F-actin in the absence of ATP causes changes in actin conformation—is a controversial one. On the one hand, Tawada (1969) observed biphasic change of birefringence of F-actin oriented by flow at different degrees of saturation by HMM, and Oosawa et al. (1973) saw change in flexibility of actin filaments when HMM bound to it. Miki et al. (1974) saw changes of anisotropy and intensity of fluorescence of ϵ-ADP that was incorporated in F-actin when it was titrated with HMM, and Miki et al. (1987) detected changes in an intermolecular distance in actin when S1 bound to it. Thomas et al. (1979) showed that binding of S1 and of HMM affected submillisecond rotational flexibility of F-actin. On the other hand, recently it has been reported that rigor binding of myosin heads has no effect on orientational (Ostap et al., 1992) distribution of actin filaments.

The objective of this work was to test whether, during muscle contraction, orientational changes occurred in actin and whether binding of HMM changed actin conformation. To this end we measured polarization of fluorescence of rhodamine-phalloidin-labeled actin filaments (Yanagida et al., 1984). Polarization of fluorescence of oriented samples carries the information about the angular distribution of fluorophores (Tregear and Mendelson, 1975; Borejdo et al., 1982; Wilson and Mendelson, 1983). In the case of F-actin, the easiest way to use oriented filaments is to make measurements in muscle fibers (Prochniewicz-Nakayama et al., 1983). However, fibers allow measurements to be taken only under isometric conditions where rotational motion may be damped because muscle is not performing work (Eisenberg and Hill, 1985). Moreover, in muscle fibers polarization signal is contributed by many filaments where averaging may obscure the effect and labeling with phalloidin is not uniform (Szczesna and Lehrer, 1993). Finally, thin filaments are not decorated uniformly with myosin heads—e.g. in a fiber the I-bands have no attached heads—and, therefore, the polarization varies along the entire length of a sarcomere (Andreev et al., 1993). For these reasons we carried out measurements on single actin filaments. We used a video technique that made it possible to compare polarized fluorescence from free

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Abbreviations used: S1, myosin-subfragment-1; HMM, heavy meromyosin; TRITC-phalloidin, tetramethyl-rhodamine-isothiocyanate-phalloidin; SD, standard deviation.

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actin filaments, from stationary single filaments to which HMM was attached, and from filaments moving during the in vitro motility assay. In contrast to muscle, individual filaments are homogeneously labeled with phalloidin; during motion they translate freely and interact with HMM uniformly along an entire length.

The results showed that, within the accuracy of the experiments (18%), the stationary and moving filaments were polarized to the same extent. Simple modeling led us to conclude that during activity actin filaments did not become more flexible and that monomers did not undergo major rotational motion. The rhodamine dipole was more disorganized in free than in bound actin filaments.

MATERIALS AND METHODS

Imaging of filaments

The video microscope used to measure the polarization of fluorescence from individual actin filaments was described previously (Borejdo and Burlacu, 1993). To observe moving filaments the light was generated by argon laser operating at 514.5 nm. The laser beam was passed through the stabilization accessory (Liconix Model 50SA, Sunnyvale, CA), which reduced the noise in the light output to less than 0.01% rms. The beam was attenuated by neutral density filters, and its direction of polarization was defined by a polarization rotator (Spectra Physics, Model 310A). The emitted light was split by a Wollaston prism that created two images of a filament. The left image was created by emitted light that was polarized horizontally, and the right one was created by light that was polarized vertically. Similar arrangement was used originally by Kinosita et al. (1991) using unpolarized excitation light and an arrangement of mirrors to split fluorescent light. The image was formed on the faceplate of an SIT camera (Cohu 5000, San Diego, CA) and recorded on a VCR (Sharp VC6610U, Mahwah, NJ).

Measuring polarization of fluorescence

Images were transferred from a VCR tape to a computer by a frame grabber (Data Translation 2861, Marlboro, MA) connected to a DT 2868 auxiliary frame processor. The relative intensities of the two images carry the information about polarization properties of sample. The intensities were analyzed by Image Pro Plus image analysis program (Media Cybernetics, Silver Spring, MD) with an accuracy better than 0.5%. Only those filaments that happened to be oriented horizontally were analyzed. Let the intensity of the light emanating from the filament being analyzed be I. Let the subscript v or h in front of the intensity denote the direction of the excitation polarization (v for vertical or perpendicular to axis of filament, h for horizontal or parallel to axis of filament). Let the subscript v or h after the intensity indicate the direction of emission polarization. When the excitation polarizer was vertical, the intensity of filaments in a part of an image that was created by vertically polarized light was ${}_{v}I_{v}$, and the intensity of the same filament in a part of an image that was created by horizontally polarized light was ${}_{v}I_{h}$. When the excitation polarizer was horizontal, the corresponding intensities were ${}_{h}I_{v}$ and ${}_{h}I_{h}$. Horizontal and vertical polarization of fluorescence were defined as $P_h = ({}_{h}I_{h} - {}_{h}I_{v})/({}_{h}I_{h} + {}_{h}I_{v})$ and $P_v = ({}_{v}I_{v} - {}_{v}I_{h})/({}_{v}I_{v} + {}_{v}I_{h})$.

Corrections for polarization of fluorescence

A systematic error in observed polarization is caused by the depolarization of emitted light arising from the high numerical aperture of the optics. The N.A. of our objective was 1.3, which gives half angle of the cone of light collected by objective as approximately $\alpha = 56°$ (we used Zeiss immersion oil 518C, $n = 1.518$). As shown by Axelrod (1979) this high N.A. produces depolarization of light such that when an object is illuminated with light polarized parallel to its axis, ${}_{h}I_{h}(\text{observed}) = K_c \cdot {}_{h}I_{h} + (K_a + K_b) \cdot {}_{h}I_{v}$ and ${}_{h}I_{v}(\text{observed}) = K_b \cdot {}_{h}I_{h} + (K_a + K_c) \cdot {}_{h}I_{v}$, where ${}_{h}I_{h}$ and ${}_{h}I_{v}$ are true parallel polarization intensities. When an object is illuminated with light polarized perpendicular to its axis, ${}_{v}I_{h}(\text{observed}) = K_c \cdot {}_{v}I_{h} + (K_a + K_b) \cdot {}_{v}I_{v}$, and ${}_{v}I_{v}(\text{observed}) = K_b \cdot {}_{v}I_{h} + (K_a + K_c) \cdot {}_{v}I_{v}$, where again ${}_{v}I_{h}$ and ${}_{v}I_{v}$ are true parallel polarization intensities. Coefficients K are given by Axelrod as: $K_a = \frac{1}{3}(2 - 3\cos\alpha + \cos^3\alpha)$, $K_b = \frac{1}{12}(1 - 3\cos\alpha + 3\cos^2\alpha - \cos^3\alpha)$ and $K_c = \frac{1}{4}(5 - 3\cos\alpha - \cos^2\alpha - \cos^3\alpha)$, where α is a half angle of the cone of light collected by the objective ($=56°$). A simple calculation shows that true P_h and P_v were underestimated in our experiment by factor $\Omega = 0.41$. Depolarization of the excitation light by high N.A. of objective was neglected.

Another source of errors in P is caused by the depolarization of light by the dichroic mirror. This results in uneven splitting of polarization components by the Wollaston prism and requires introduction of correction factors c_v and c_h. Correction factors were calculated by measuring in microscope polarization of fluorescence of rhodamine B dye. Polarization of small dye in solution must be 0 and must be independent of excitation polarization. In our optical system c_v and c_h were 1.50 and 1.00, respectively.

Finally, correction is necessary because background was non-zero due to fluorescence of the glass coverslip, imperfect separation of excited and emitted light by the dichroic cube assembly of Zeiss Standard microscope, residual free phalloidin, and dark noise of the SIT camera. Let ${}_{h}I_{hb}$ indicate the average intensity of the background near a filament in an image obtained with horizontal excitation and created by horizontally polarized light. Let the average intensity of the background in the remaining three images of the same filament be ${}_{h}I_{vb}$, ${}_{v}I_{vb}$, and ${}_{v}I_{hb}$. Then the corrected polarizations are

$$P_h = \frac{(1 + \Omega)[({}_{h}I_{h} - {}_{h}I_{hb}) - ({}_{h}I_{v} - {}_{h}I_{vb})/c_h]}{({}_{h}I_{h} - {}_{h}I_{hb}) + ({}_{h}I_{v} - {}_{h}I_{vb})/c_h} \quad (1)$$

$$P_v = \frac{(1 + \Omega)[({}_{v}I_{v} - {}_{v}I_{vb})/c_v - ({}_{v}I_{h} - {}_{v}I_{hb})]}{({}_{v}I_{v} - {}_{v}I_{vb})/c_v + ({}_{v}I_{h} - {}_{v}I_{hb})}. \quad (2)$$

The errors due to photobleaching were neglected because all solutions contained deoxygenating solution (Englander et al., 1987) including 50 mM DTT, and because the laser light intensity was low (incident power at 514.5 nm was typically 0.15 mW). Errors due to smearing of image of moving filaments were neglected because, for Burle 4804H silicon target tube (employed in Cohu 5000 SIT camera), typically 6–8% of the signal in a given frame is contributed by an event that occurred in the preceding frame (33 ms earlier). Assuming that in 33 ms average filament translated by 20% of its length (an overestimation), only $7\% \times 0.20 = 1.4\%$ of a signal in a given frame is contributed by events that occurred in a previous frame.

Materials

Tetramethyl-rhodamine-isothiocyanate-phalloidin (TRITC-phalloidin), DTT, ATP, and ADP were from Sigma Chemical Co. (St. Louis, MO).

Proteins

Myosin was prepared from rabbit skeletal muscle by the method of Tonomura et al. (1966). HMM was obtained by a chymotryptic digestion of myosin according to Weeds and Pope (1977). Skeletal actin was prepared according to Spudich and Watt (1971). The concentrations of proteins were measured by absorbance using: G-actin, $A^{1\%}(290) = 6.3$; F-actin, $A^{1\%}(290) = 6.7$; HMM, $A^{1\%}(280) = 6.47$. Proteins were checked for purity by SDS-PAGE and for activity by measuring actin-activated Mg-ATPase. Stock solution of HMM was 8.5 mg/ml and of actin about 5 mg/ml. F-actin was labeled with TRITC-phalloidin by diluting protein with buffer containing 25 mM K-Acetate, 2 mM Mg-Acetate, 1 mM EGTA, 25 mM TRIS-Acetate, pH 7.5, to 1 μM and adding equimolar concentration of the dye and incubating overnight on ice.

Solutions

0.1 mg of TRITC-phalloidin was dissolved in 0.1 ml of 50% methanol and diluted 10 times with 25 mM K-Acetate, 25 mM TRIS-Acetate, pH 7.5.

Motility was measured in an A-buffer containing 25 mM K-Acetate, 50 mM DTT, 2 mM MgAc, 1 mM EGTA, 25 mM TRIS-Acetate, pH 7.5, and oxygen scavenging enzymes (Englander et al., 1987).

RESULTS

Filaments in rigor

We first looked at filaments immobilized by binding to an HMM-coated coverslip. Coverslips were coated with 0.1% nitrocellulose; 5 μl of A-buffer containing 1.2 μM HMM and 10 nM TRITC-phalloidin-F-actin was placed on a coverslip. Fig. 1 shows a typical image of actin filaments in the absence of ATP. In *A* the excitation light was polarized horizontally. The emitted light that was polarized horizontally created an image at the left of the frame, and the emitted light that was polarized vertically created an image at the right of the frame. At the center the two images overlap, creating background with higher intensity. Care was taken not to analyze filaments present in this area. Only the filaments with long axis oriented horizontally and outside of overlap area were considered. One such filament is pointed to by arrows. The intensity of a filament was measured by Image Pro by enclosing it with a rectangular Area-Of-Interest (AOI) and measuring the average intensity within it. This intensity was ${}_{h}I_{h}$. The average intensity of the background near this filament was ${}_{h}I_{hb}$. The average intensity of the same filament in the right image was ${}_{h}I_{v}$. The average intensity of the background near this filament was ${}_{h}I_{vb}$. Horizontal polarization of fluorescence, P_h, was then calculated according to Eq. 1. In *B* the excitation light was polarized vertically. Arrows point to a filament that was horizontal (it is the same filament as in *A*). The average intensity of this filament in the left image was ${}_{v}I_{h}$. The average intensity of the background near this filament was ${}_{v}I_{hb}$. The average intensity of the same filament in the right image was ${}_{v}I_{v}$. The average intensity of the background near this filament was ${}_{v}I_{vb}$. Vertical polarization of fluorescence, P_v, was calculated according to Eq. 2. The histogram of horizontal (parallel) and vertical (perpendicular) polarizations from all experiments are shown in Fig. 3 (*open squares*). It is a plot of the frequency with which a particular value of polarization occurred versus the value of this polarization. Results are summarized in Table 1. In the absence of ATP, in 47 experiments in which P_h was measured, the average value was 0.196 ± 0.018, and in the 71 experiments in which P_v was measured the average value was 0.011 ± 0.021. The data was analyzed by fitting to two independent models (see Discussion). In the first, the helix-random model, a fraction σ of probe molecules is thought to describe a perfect helical arrays around F-actin characterized by a polar angle Θ_H. The remaining fraction, 1-σ, is oriented randomly ((Tregear and Mendelson, 1975), Mendelson and Morales (see Borejdo and Putnam, 1977), Borejdo et al., 1982), Wilson and Mendelson, 1983). In the second, the Gaussian model, the disorder of the probe angle Θ is thought to spread in a Gaussian manner around the mean value Θ_G with a standard deviation δ (Thomas and Cooke, 1980; Wilson and Mendelson, 1983; Yanagida, 1981; Barnett et al., 1986). The average angles Θ as well as disorder parameters σ and δ were calculated and are given in Table 2. It is seen that both models give identical values of Θ, and agree that in rigor disorder is small. Neither model takes into account changes in azimuthal angle.

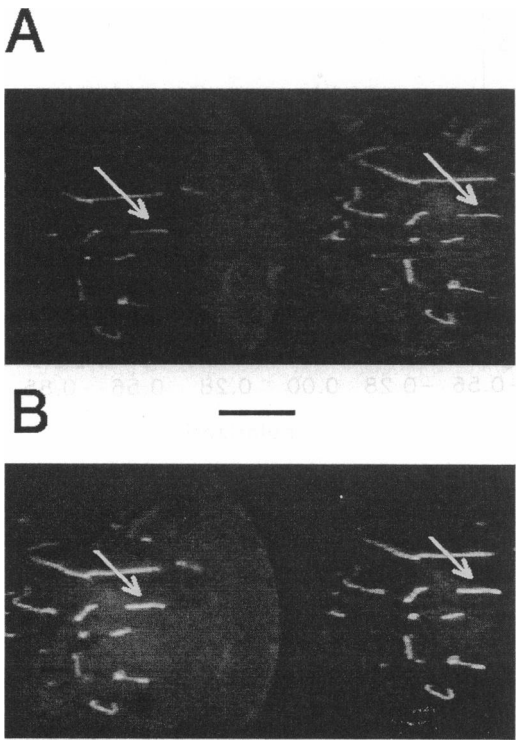

FIGURE 1 Fluorescence image of actin filaments immobilized by binding to coverslip-adsorbed HMM. 10 nM TRITC-phalloidin-F-actin and 1.2 μM HMM in A-buffer. (*A*) Excitation polarization horizontal. Arrows point to a filament that happened to be oriented horizontally. (*B*) Excitation polarization vertical. The center of each frame is brighter, because in this area horizontally and vertically polarized emitted beams overlap. Filaments lying in this area were not measured. Bar is 10 μm.

Moving filaments

To induce motion, samples were prepared in the same fashion as in rigor, but solutions contained, in addition, 2 mM ATP. The motion began a few minutes after placing the coverslip on the microscope slide, probably because HMM needed a few minutes to get adsorbed to nitrocellulose. Filaments moved with an average velocity of 6 μm/s (SEM = 2 μm/s). Fig. 2 shows the image of moving filaments. Now

TABLE 1 Polarized fluorescence of stationary, moving, and free actin filaments

	P_h[a]	P_v[a]	n[b]
Rigor	0.196 ± 0.018	0.011 ± 0.021	47, 71
+ATP	0.225 ± 0.110	0.039 ± 0.022	61, 62
Free[c]	0.238 ± 0.022	0.256 ± 0.021	15, 12

[a] Mean ± SEM.
[b] Number of measurements of P_h, P_v.
[c] Determined from a section of a filament that was not attached to the coverslip.

TABLE 2 Mean angles (Θ_H, Θ_G) and degree of randomness (σ, δ) of helix-random and Gaussian models of arrangement of dye around actin filaments

	Helix-random		Gaussian	
	Θ_H[a]	σ[b]	Θ_G[c]	δ[d]
Rigor	49.3	0.99	49.3	2.8
+ATP	48.9	0.92	49.1	8.9
Free[e]	51.5	0.65	51.4	19.3

[a] Angle, in degrees, of perfectly helical fraction in helix-random model.
[b] Fraction having perfect helical symmetry in helix-random model.
[c] Mean, in degrees, of Gaussian distribution of angles in Gaussian model.
[d] Standard deviation, in degrees, of the mean in Gaussian model.
[e] Determined from a section of a filament that was not attached to the coverslip.

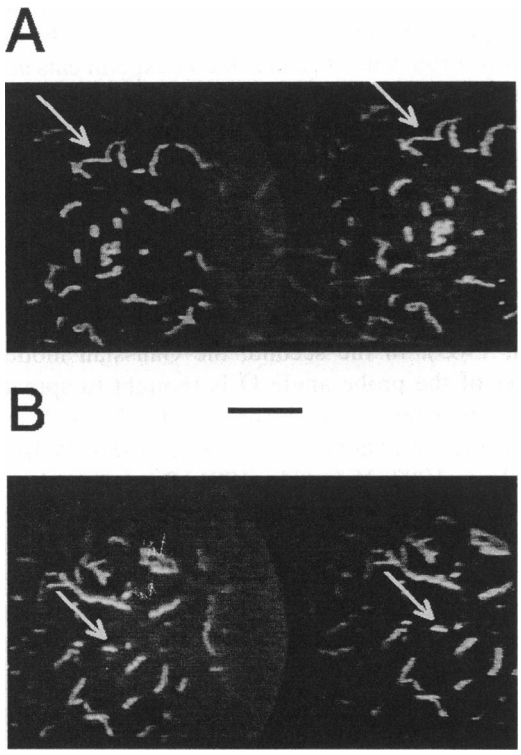

FIGURE 2 Fluorescence image of moving actin filaments. 10 nM TRITC-phalloidin-F-actin, 1.2 μM HMM, 2 mM ATP in A-buffer. (*A*) Excitation polarization horizontal. Arrows point to a filament that had a large segment that, at this instant, was oriented horizontally. (*B*) Excitation polarization vertical. Arrows point to a filament that happened, at this instant, to be oriented horizontally. Bar is 10 μm.

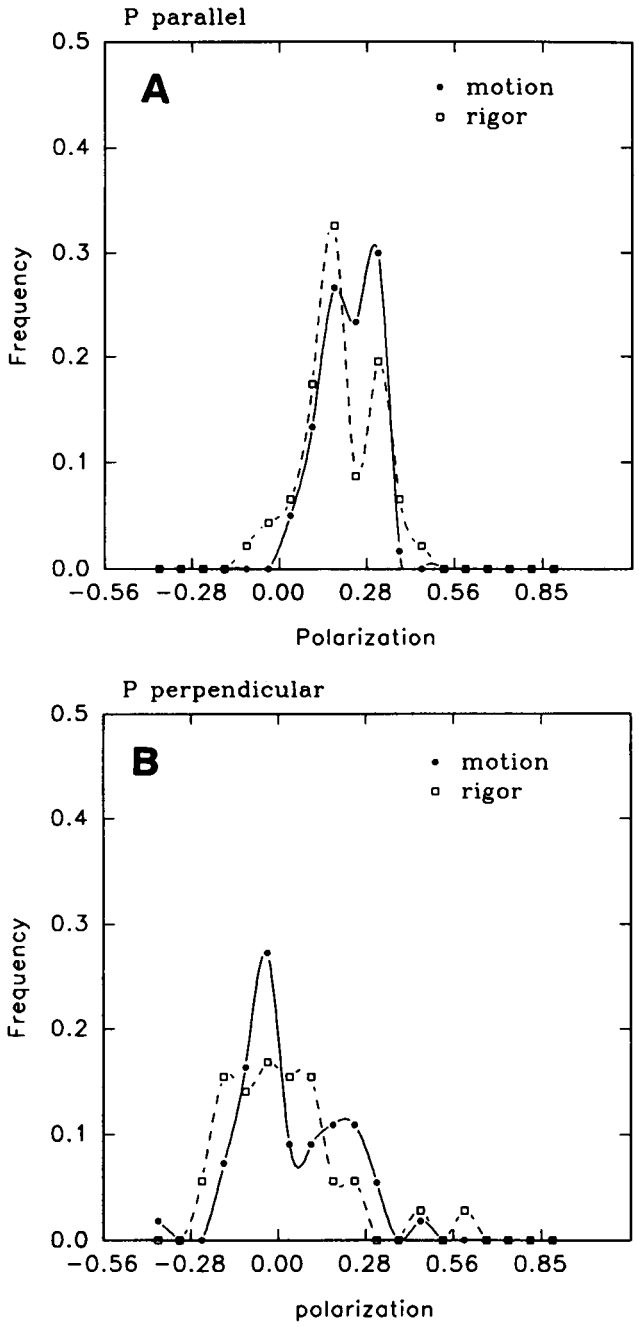

FIGURE 3 Histogram of values of polarization of fluorescence of stationary actin filaments and of filaments during motility assay. (*A*) Parallel excitation. (*B*) Perpendicular excitation. The data points were fitted with a spline function. Open squares: filaments in rigor; filled circles: moving filaments.

different images were obtained when filaments were illuminated with horizontally polarized light (Fig. 2 *A*) and with vertically polarized light (Fig. 2 *B*) because about 20 s elapsed between recordings. When filaments were moving, the measurements of polarization were more difficult: filaments were often bent, and only a part of a filament, such as the part pointed to in Fig. 2 *A*, was horizontal. Further, many filaments (such as the one pointed to in Fig. 2 *B*) were very short. The distribution of polarizations is shown in Fig. 3 (*filled circles*). Now in 61 experiments the average value of P_h was 0.225 ± 0.110, and in 62 experiments the average value of P_v was 0.039 ± 0.022. The difficulty in measuring polarizations of moving filaments is reflected in the fact that now SEM of measurement is about 50% of the mean (P_h, Table 1). t-test revealed no statistically significant difference between polarizations (see Discussion). Both models indicate that the average angle changed little from the average angle in rigor, and agree that during motion the amount of disorder increased slightly (Table 2).

Effect of binding of HMM

To test whether binding of actin filaments to HMM adsorbed to nitrocellulose changes orientation of actin protomers, we compared polarization of fluorescence of filaments immobilized by binding to HMM and filaments free in solution. Filaments were attached to a surface of borosilicate glass coverslips (without nitrocellulose coating) through at least two points. The central section of filaments was free, as evidenced by the fact that it executed Brownian motion. Only the section of filaments that happened to be in focus and that was oriented horizontally was analyzed. One such section is pointed to by arrows in Fig. 4. The pictures are now quite different than images of immobilized filaments (e.g., Fig. 1): analysis of Fig. 4 *A* shows that the intensity of filament at left is now greater than the intensity of the same filament at right, suggesting that P_h is now large. Similarly, analysis of Fig. 4 *B* shows that the intensity of filament at left is now smaller than the intensity of the same filament at right, suggesting that P_v is also large. Quantitative analyses of all experiments are shown in histograms of Fig. 5 *A* and *B*. In 15 experiments in which P_h was measured, the average value was 0.238, and in 12 experiments in which P_v was measured the average value was 0.256 (Table 1). There was no statistically significant difference between polarizations. Both models (Table 2) indicated a significant disorganization of the dye. Significance of this finding is dealt with in Discussion.

Steady-state (Perrin) plots of TRITC-phalloidin-F-actin

To use polarization of fluorescence data to construct a model of arrangement of the dye on the surface of F-actin (see Discussion), it is necessary to calculate the angle between the absorption and emission dipoles of rhodamine, and the rigidity of the attachment of TRITC-phalloidin to actin. Measuring the polarization of fluorescence of actin filaments in solution at different degrees of immobilization of the dye (Perrin plot) allows such calculation. Let an absorption dipole of the dye be able to rotate on the surface of actin through a cone with characteristic angle α. The "true" and "false" limiting polarizations of fluorescence were p_o = 0.381 and p_x = 0.366 (Fig. 6). The ratio of limiting polarizations gave $\alpha = p_x/p_o = (3\cos^2\alpha - 1)/2 = 9.2°$ (Weber,

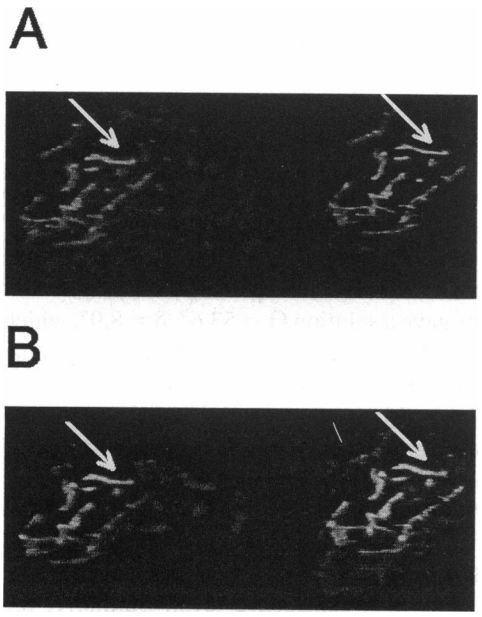

FIGURE 4 Fluorescence image of "free" actin filaments. 10 nM TRITC-phalloidin-F-actin. (*A*) Excitation polarization horizontal. Arrows point to a section of a filament that happened to be horizontal and in focus. (*B*) Excitation polarization vertical. Magnification as in Fig. 1.

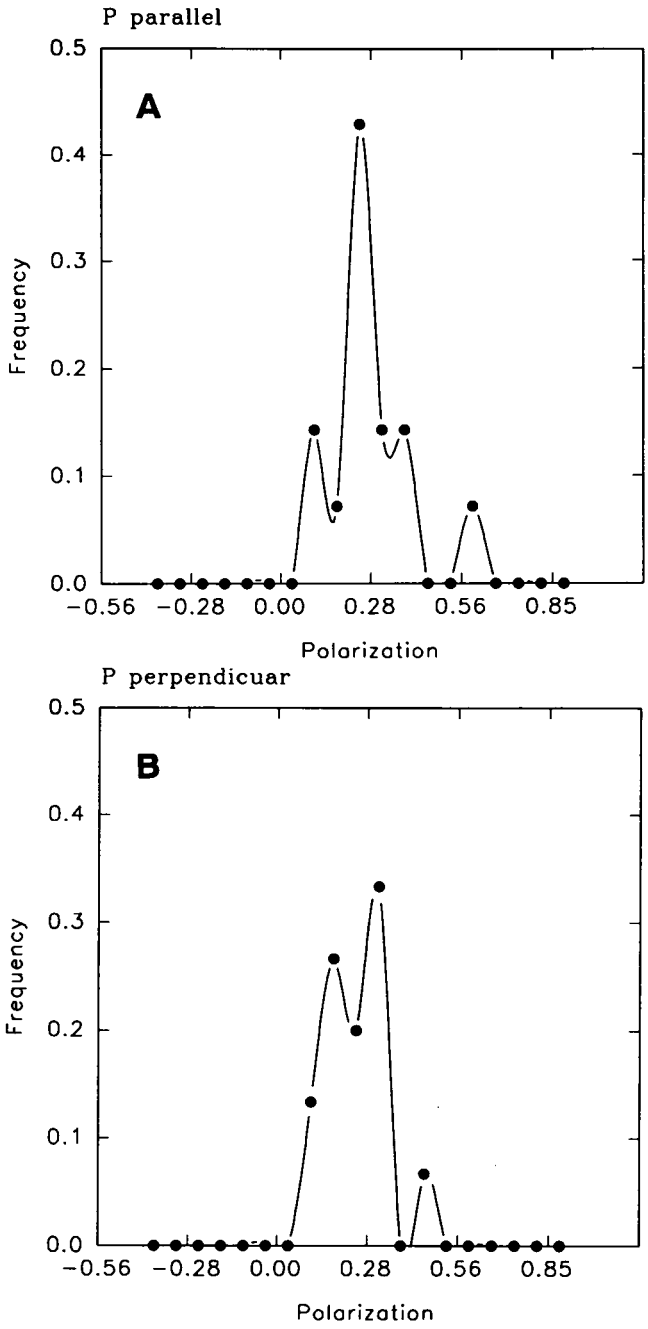

FIGURE 5 Histogram of values of polarization of fluorescence of actin filaments free in solution. (*A*) Parallel excitation. (*B*) Perpendicular excitation. The data points were fitted with a spline function.

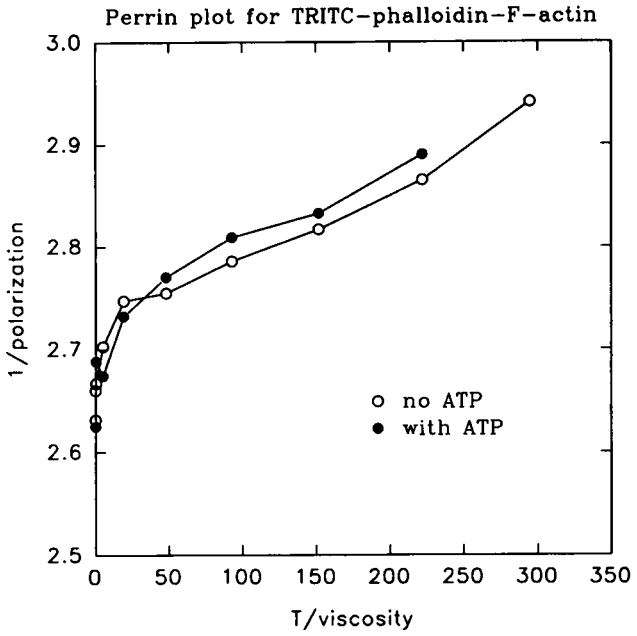

FIGURE 6 Perrin plots of F-actin labeled with IATR-phalloidin in the absence (○) and in the presence (●) of 2 mM ATP. 0.5 μM IATR-phalloidin-F-actin in buffer A containing 10–90% sucrose. Measurements were done in SLM 500C spectrofluorimeter at 20°C. The excitation-emission wavelengths were 514–575 nm; bandpass filter was #5. Vertical scale: 1/polarization of fluorescence; horizontal scale: ratio of absolute temperature to viscosity in degrees Kelvin/centipoise.

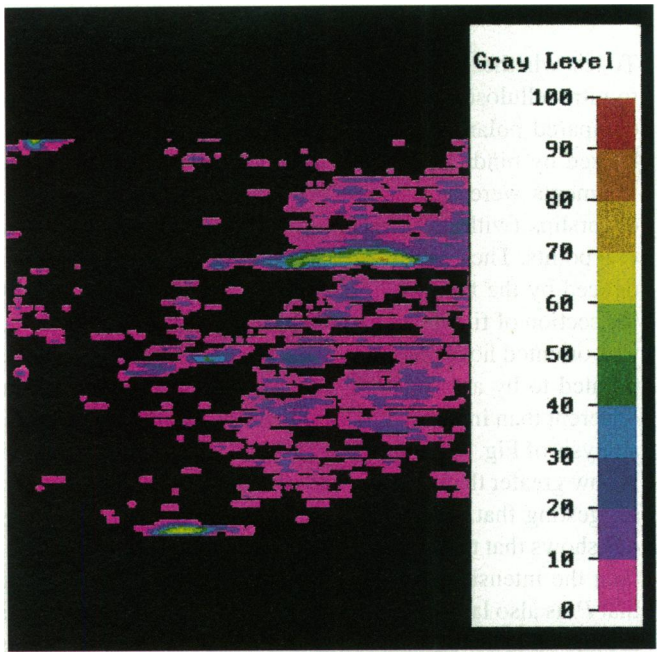

FIGURE 7 Horizontal polarization image of actin filament. Every pixel of a pair of images of a filament pointed to by arrows in Fig. 1 *A* was arithmetically manipulated according to Eq. 1. Small number (10) was added to the denominator of Eq. 1, to avoid dividing by 0 at the black areas.

1966). The angle between the absorption and emission dipoles of rhodamine λ is equal to $\cos^{-1}(\mathrm{Sqrt}((3p_o + 1)/(3 - p_o)))$ and is 25.2°.

It is possible that any changes in polarization occurring upon addition of ATP are due to direct effect of nucleotide on the mobility of the dye. To test for this possibility, we measured Perrin's plots of rhodamine-phalloidin-F-actin both in the absence (Fig. 6, *open circles*) and in the presence (Fig. 6, *filled circles*) of ATP. ATP made no difference to Perrin's plots, suggesting that the nucleotide had no effect on mobility of rhodamine.

Polarization imaging

The distribution of polarizations within a single actin filament can be visualized by constructing a polarization image. This can be done only for filaments that happen to lie on a coverslip with their long axes aligned horizontally (or vertically), such as the pair pointed to by arrows in Fig. 1 *A*. For every pixel in the pair of images, a value $[({}_hI_h - {}_hI_{hb}) - ({}_hI_v - {}_hI_{vb})/c_h)]/[({}_hI_h - {}_hI_{hb}) + ({}_hI_v - {}_hI_{vb})/c_h)]$ was calculated. The result for a pair of filaments pointed to in Fig. 1 *A* is shown in Fig. 7. Because both ${}_hI_h$ and ${}_hI_v$ were exact replicas of the same object (except for the emission polarizations) and were obtained simultaneously, the errors due to the focusing, to uneven spatial distribution of the exciting light, and to the fluctuations in the laser power were eliminated. Both images of a filament were carefully registered to make sure that coordinates of pixels of horizontal image correspond exactly to the coordinates of pixels of the vertical image. The result of computations was multiplied by 200 to bring the polarizations within 8-bit gray level range ($0 \leq$ Gray level ≤ 255). Thus, gray level of, e.g., 70 corresponds to polarization value of 0.350.

The characteristic feature of polarization image of actin filament was that polarization was not constant along its length. Polarization was always higher at the center of the filament. This may reflect better immobilization of actin monomers at the center.

DISCUSSION

The accuracy of measurements can be estimated by calculating angles from four pairs of polarizations: (P_h – SD, P_v + SD) and (P_h + SD, P_v – SD) for rigor and moving filaments. Of the four, the pair P_h – SD, P_v + SD for rigor filaments gave a solution $\Theta = 53.6°$, $\delta = 8.9°$, which differed most from the average value. From this we estimate that Θ can be determined with an accuracy of $2 \cdot (53.6 - 49.3) = 8.6°$. Within this accuracy, polarizations of fluorescence of transition dipoles of phalloidin attached to actin filaments were not statistically different when filaments were stationary or were moving in the in vitro motility assay. According to nonpaired t-test, the average P_h for stationary filaments was not different from average P_h for moving filaments, with $t = 0.14$ at 15% confidence level. Similarly, for vertical polarization, the average P_v for stationary filaments was not statistically different from average P_v for moving filaments with, $t = 0.91$ at 34% confidence level. We conclude that we can determine the difference in angles to within about 9° with

at least 15% confidence. Results also indicate that binding of filaments to immobilized HMM made a significant difference to vertical polarization (Table 1).

To express these finding in terms of reorientation of actin monomers, it is necessary to postulate a model of arrangement of the dye on the surface of F-actin. We use here two types of arrangement. (i) helix-random model: in this model a fraction of probe molecules is thought to describe perfect helical array (characterized by polar angle Θ_H) around F-actin, and the remaining fraction is randomly oriented. The arrangement is the same as the one used for myosin in skeletal muscle fiber by Tregear and Mendelson (1975), Mendelson and Morales (see Borejdo and Putnam, 1977), and Borejdo et al. (1982) because the arrangement of actin monomers in filaments, like myosin in thick filaments, exhibits helical symmetry. The total disorder can be characterized by a parameter $1 - \sigma$, which is the sum of the disorder introduced by nonspecific labeling, skew of actin filaments (Yanagida and Oosawa, 1978), dye motion on the surface of actin, and the presence of free phalloidin. (ii) Gaussian model: the dipoles are thought to be spread with Gaussian probability around the mean value Θ_G with a standard deviation δ (Thomas and Cooke, 1980; Wilson and Mendelson, 1983; Yanagida, 1981; Barnett et al., 1986).

We have shown (Fig. 6) that the rhodamine dye is well immobilized on the surface of actin filaments and that absorption and emission dipoles are separated by a relatively small angle. It can be shown for helix-random model (Mendelson and Morales, see Borejdo and Putnam, 1977) that in such a case and with $\psi = 360°$ (ψ is the angle between excitation and observation directions) polarizations can be approximated by

$$P_v = 15\sigma(3\sin^4\Theta - 4\cos^2\Theta\sin^2\Theta) + \frac{16(1-\sigma)}{15\sigma(3\sin^4\Theta + 4\cos^2\Theta\sin^2\Theta)} + 32(1-\sigma)$$

$$P_h = 15\sigma(\cos^4\Theta - 0.5\cos^2\Theta\sin^2\Theta) + \frac{2(1-\sigma)}{15\sigma(\cos^4\Theta + 0.5\cos^2\Theta\sin^2\Theta)} + 4(1-\sigma).$$

For filaments in rigor, the exact solution of these equations was obtained by *Mathematica 2.1* with starting values $\Theta = 15°$ and $\sigma = 0.4$. After 15 iterations by Newton's method, a unique solution $\Theta = 49.3°$ and $\sigma = 0.99$ was found (Table 2). This shows that in the absence of ATP, a model in which all fluorophores were arranged with perfect helical symmetry along actin axis satisfactorily describes the arrangement of the dye. In this model, absorption (or emission) dipole of the dye forms an angle of about 49° with respect to filament axis. High degree of order was also observed in skeletal muscle in rigor (Wilson and Mendelson, 1983; Borejdo et al., 1982).

For the Gaussian model we consider a system where dye dipoles are oriented with respect to the long axis of actin filaments at mean polar angle Θ_0 with standard deviation δ. The probability for dipole orientation at any angle Θ is given by Gaussian $\rho(\Theta) = \exp[-(\Theta - \Theta_G)^2/2\delta^2]$. The polarizations P_v and P_h are expressed as functions of the averages $S_2 = \langle\sin^2\Theta\rangle$ and $S_4 = \langle\sin^4\Theta\rangle$

$$P_v = \frac{7S_4 - 4S_2}{4S_2 - S_4} \qquad P_h = \frac{2 - 5S_2 + 3S_4}{2 - 3S_2 + S_4}$$

S_2 and S_4 are given, e.g., by Wilson and Mendelson (1983). For filaments in rigor, the exact solution $\Theta = 49.3°$ and $\delta = 2.8°$ was obtained by *Mathematica* with starting values $\Theta = 10°$ and $\delta = 0.1°$ (Table 2). This confirms the conclusion reached from helix-random model that in the absence of ATP the absorption (or emission) dipole of the dye formed an angle of about 49° with respect to filament axis, and that dipoles were arranged with excellent symmetry relative to actin axis.

When filaments were moving, the solution was $\Theta = 48.9°$, $\sigma = 0.92$ for helix-random model and $\Theta = 49.1°$, $\delta = 8.9°$ for Gaussian model. We conclude, with high degree of confidence, that during motion the average angle of the absorption (or emission) dipole of the dye did not change. During motion the fluorophores underwent small disorganization as reflected by an increase in $1\text{-}\sigma$ and δ. It is unlikely that this small disorganization was due to mobilization of rhodamine dye by ATP, because ATP made little difference to Perrin's plots (Fig. 6). Disorganization may be due to rapid rotations confined to a narrow cone. But if such rotations exist, they must occur with a rate faster than $(33\ \text{ms})^{-1}$ (video rate) and be confined to a cone smaller than about 6° (Table 2). Alternatively, the observed increase may be related to the fact that in free filaments disorganization is large (Table 2 and see below) and that in the presence of ATP filaments interact with far fewer HMM molecules than in rigor (Uyeda et al., 1990).

Our findings are consistent with the results of Kinosita et al. (1991), who were the first to use dual-view microscopy to compare polarizations of immobilized and moving actin filaments. They observed no change in the polar angle of rhodamine-phalloidin bound to actin. Our result asserting with at least 15% confidence that the orientation of actin protomers during motion differs by less than 9° from the orientation in rigor seems to be in conflict with the results of Oosawa et al. (1973), who suggested that actin undergoes changes in flexibility during muscle contraction. However, it is difficult to assess the degree of disagreement (if any) because Oosawa et al. (1973) did not estimate angular change of actin protomers. Our conclusion is inconsistent with the results of Yanagida (1981), who showed that the transition dipole of ϵ-ADP incorporated into F-actin of striated muscle fibers changes orientation upon activation. Finally, our results are inconsistent with the suggestion of Schutt and Lindberg (1992) that thin filaments undergo gross conformational change during contraction.

Our results do not exclude the possibility that motion results in azimuthal rotation of actin monomers. Such rotations could occur, for example, if actin always presented the same face towards the plane of the coverslip. In fact, there is evidence that this type of motion occurs (Nishizaka et al., 1993).

It is unlikely that unlabeled actin, in contrast to phalloidin-labeled actin, undergoes rotational motions during movement. This is because native and phalloidin-labeled actins differ little in contractile properties. Labeled filaments move in the in vitro motility assay as fast as in native muscle (Kron and Spudich, 1986). Labeling changes polymerizability of modified actin only marginally (Miki et al., 1987), and has no effect on distribution of lengths of F-actin (Burlacu et al., 1992) or on tension or Ca^{2+} regulation of striated muscle (Prochniewicz-Nakayama et al., 1983). In fact, it has been speculated that labeling with phalloidin has no deleterious effects on F-actin in systems in which polymerization-depolymerization of actin plays no role in function (Prochniewicz-Nakayama et al., 1983). (We note parenthetically that we attempted to visualize filaments by labeling actin at Cys-374 with IATR. It is known that labeling actin at this position with 5-iodoacetamide fluorescein (Wang and Taylor, 1980), with pyrene (Kouyama and Mihashi, 1980; Cooper et al., 1983), or with IATR (Tait and Frieden, 1982) does not grossly affect properties of actin. Cys-374-labeled filaments could be visualized in the optical microscope, but only for a few seconds before their length decreased below the resolution of the microscope. The length decreased because observations were made at a nanomolar concentration (Burlacu et al., 1992), 100-fold lower than the critical concentration of F-actin.)

Effect of binding of HMM

The results showed that the average angle that the transition dipole of the dye formed with actin axis was different when filaments were immobilized by binding to HMM that was adsorbed to the coverslip and when they were free in solution ($t = 1.26, P = 0.21$ for P_h; $t = 5.26, P = 1.0 \times 10^{-6}$ for P_v). The difference was particularly large for P_v, despite the fact that only a small number of measurements were done for free actin (because it was difficult to find filaments that were in focus over an entire length). It is unlikely that difference was due to the contributions from out-of-focus segments of filament, because variance of polarization of free actin (Table 1) was small. This effect of HMM on conformation of F-actin is consistent with earlier results of Tawada (1969), Oosawa et al. (1973), Miki et al. (1974, 1987), Yanagida and Oosawa (1978), and Thomas et al. (1979), but conflicts with recent results of Ostap et al. (1992). The fact that rhodamine dipole was more disorganized in free than in bound actin is consistent with the results of Egelman and DeRosier (1992), who recently confirmed their earlier claim that actin monomers in a filament exhibit random disorder (of 5–6° magnitude).

Polarization imaging

The values reported in Table 1 are the average polarizations of the whole filament. But in muscle, polarization can vary along the length of a sarcomere (Andreev et al., 1993). Measuring polarization of fluorescence of every pixel in an image is a convenient way to obtain spatially resolved images (Dix and Verkman; Fushimi et al., 1990; Gough and Taylor, 1993). Spatially resolved images of actin filaments in rigor (e.g., Fig. 7) show that polarization is greater at the center of a filament than at the ends. Because phallodin binds uniformly to isolated filaments (Szczesna and Lehrer, 1993), this may be due to imperfect immobilization of filaments by HMM near the ends.

Conclusion

Our results suggest that mechanochemical interactions between myosin heads and actin do not deform the polymer structure of actin filaments. S1 reorientations observed during contraction (Borejdo et al., 1979; Stein et al., 1990; Berger and Thomas, 1993) were most likely confined solely to the myosin heads. Large disorganization seen in actin filaments in solution disappeared upon binding of myosin heads.

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