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Fluorescent actin filaments move on myosin fixed to a glass surface

(energy transduction/muscle/motility assay/*Dictyostelium*)

STEPHEN J. KRON AND JAMES A. SPUDICH

Department of Cell Biology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **Single actin filaments stabilized with fluorescent phalloidin exhibit ATP-dependent movement on myosin filaments fixed to a surface. At pH 7.4 and 24°C, the rates of movement average 3–4 μm/s with skeletal muscle myosin and 1–2 μm/s with *Dictyostelium* myosin. These rates are very similar to those measured in our previous myosin movement assays. The rates of movement are relatively independent of the type of actin used. The filament velocity shows a broad pH optimum between 7.0 and 9.0, and the concentration of ATP required for half-maximal velocity is 50 μM. Evidence was obtained to suggest that movement of actin over myosin requires at most the number of heads in a single thick filament. This system provides a practical, quantitative myosin-movement assay with purified proteins.**

The lack of a simple *in vitro* assay for myosin movement has hampered progress in elucidating the mechanism by which actin and myosin couple ATP hydrolysis to mechanical work. We have sought to develop a totally purified system that would permit quantitative determination of the rate of myosin movement along actin.

Several systems for observing movement of purified actin and myosin have been reported (1–6). The first quantitative measurement of rates of movement of purified myosin along actin *in vitro* were made by using the *Nitella*-based movement assay of Sheetz and Spudich (7). In that assay, myosin filaments are attached to polystyrene beads, and these myosin beads are deposited on the cytoplasmic face of a dissected *Nitella axillaris* cell. These cells contain rows of chloroplasts attached to the inner face of the cell membrane. Bundles of actin filaments of uniform polarity lie on these chloroplast rows (8). Myosin beads that come into contact with the actin bundles move unidirectionally over long distances in the same direction as cytoplasmic flow in the unopened cell. The rates of movement of the beads can be determined by noting position and time elapsed at several points along the bead path. The *Nitella*-based assay yields reproducible and quantitative rate data with relatively small samples of purified myosin. Nonetheless, this assay is flawed because it depends on the biochemically undefined actin cables of the *Nitella* cell, which are stabilized by unknown factors and may be contaminated by components of the *Nitella* cytoplasm. Thus, we have worked to replace the *Nitella*-based assay with a movement assay using only purified proteins.

The *Nitella*-based assay demonstrated a principle that we have exploited in designing movement systems composed of purified proteins. Although the myosin filaments are bipolar and are bound to the beads in random orientations (9), the beads move in only a single direction—that defined by the polarity of the actin filaments. This suggests that only those myosin cross-bridges oriented properly can interact productively with an actin filament. Those not oriented correctly presumably cannot recognize their binding site on actin. Thus, the direction of movement is defined by the polarity of the actin filament.

Our first approach to establish a purified movement system (5) was to replace the *Nitella* actin cables with an array of polar, aligned actin filaments bound to the substrate by the *Dictyostelium* protein severin (10). In the presence of Ca^{2+}, this protein severs actin filaments and becomes bound to their barbed ends irreversibly. Severin was biotinylated and bound to filamentous actin. The biotin-severin-actin was applied in the presence of flow to an avidin-coated substrate, producing an array of aligned filaments with their free pointed ends pointing downstream. As predicted, myosin beads placed on such arrays moved toward the barbed ends of the filaments, opposite to the direction of buffer flow. The rates of movement of these myosin beads were similar to those measured with the *Nitella* assay. These experiments provided quantitative *in vitro* measurement of movement of purified actin and myosin. However, this system has been difficult to develop into a practical myosin movement assay. Many beads attach to the substratum without moving, and those that move do so for relatively short distances. Good movement may require a large area of well-oriented actin filaments, which is difficult to produce. Therefore, we sought to develop a new approach that would remove the requirement for large arrays of actin with known polarity.

Yanagida *et al.* (4) observed single fluorescent actin filaments in solution by using a video light microscope. They found that the amplitude and frequency of bending of the filaments increased in the presence of soluble myosin fragments and ATP. We considered that we might observe linear movement of single fluorescent actin filaments along myosin filaments by inverting our movement system, immobilizing the myosin on the substrate, and allowing single actin filaments to attach to the bound myosin.

In this study, we report that, in the presence of ATP, myosin filaments attached to glass are indeed capable of supporting movement of single actin filaments labeled with rhodamine phalloidin. The rates of movement of these single actin filaments are consistent with those measured in our previous assays and depend on the concentration of ATP, the buffer pH, and the type of myosin.

MATERIALS AND METHODS

Rabbit skeletal-muscle actin was purified by the method of Spudich and Watt (11) and recycled as described by Pardee and Spudich (12). Rabbit skeletal muscle myosin was purified by the method of Kielly and Harrington (13) and stored at −20°C in 50% (vol/vol) glycerol. Myosin from *Dictyostelium discoideum* was purified as described by Mockrin and Spudich (14) and stored at −20°C in 50% glycerol. *Dictyostelium* actin was purified as described by Uyemura *et al.* (15) and lyophilized in low-salt buffer. Tetramethylrhodamine

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Abbreviations: Me_4R, tetramethylrhodamine; Me_4RP, Me_4R-conjugated phalloidin; EM, electron microscopy.

(Me_4R) conjugated to phalloidin (Me_4RP) was obtained from Molecular Probes (Junction City, OR). Bovine serum albumin was from Sigma. ATP was from Calbiochem–Behring.

For these experiments we used a flow cell designed for use with high-power microscope objectives (16) with valves obtained from Hamilton (Reno, NV). We used a peristaltic pump (Rainin, Woburn, MA) to pull solutions through the cell. Movement was recorded with a Zeiss standard microscope outfitted with epi-illumination optics and a Me_4R filter set (Zeiss model 48 77 15), a Planachromat ×63 objective, a Ni-Tec (Garland, TX) NVS-100 image intensifier, an RCA TC2000 Ultricon camera, and a Panasonic VHS recorder.

Purified filamentous rabbit actin was incubated on ice overnight with a molar excess of Me_4RP in standard buffer (25 mM imidazole, pH 7.4/25 mM KCl/4 mM $MgCl_2$/0.2 mM $CaCl_2$/1 mM ATP/0.5 mM 2-mercaptoethanol). The lyophilized *Dictyostelium* actin was resolubilized with distilled water and centrifuged 30 min at 450,000 × *g*, and an aliquot was added to a molar excess of Me_4RP in standard buffer. Both the rabbit and *Dictyostelium* Me_4RP-actin filaments were diluted to a final concentration of 12.5 nM actin before use in the experiments.

Myosin in high salt was diluted to 0.1–0.2 mg/ml into standard buffer and incubated on ice for >15 min. Under these conditions, myosin formed short (1 μm or less), bipolar thick filaments of relatively uniform length and diameter, as determined by electron microscopy (EM) of negatively stained specimens (data not shown).

Glass coverslips (12-mm diameter), previously cleaned in EtOH/KOH, were rinsed with distilled water and air-dried. The coverslips were placed onto 0.1- to 0.25-ml drops of myosin at 4°C for 30–60 s. For some experiments, coverslips were left in contact with the myosin for ≈15 min. The coverslips were transferred to 0.25-ml drops of bovine serum albumin (0.5 mg/ml) at 4°C for ≈5 min and then brought to 24°C, which was the temperature at which all of the experiments were performed. When EM grids were prepared in this manner and then negatively stained with uranyl acetate (1% in water), the myosin formed arrays of scattered, randomly oriented bipolar filaments (data not shown).

To begin each experiment, the flow cell was filled with standard buffer. The myosin-coated coverslips were sealed into the fluid-filled flow cell with grease (Apiezon M), and unbound protein was washed away with a flow of buffer. The flow was increased to ≈0.25 ml/min, and 0.5 ml of the diluted Me_4RP-actin filaments were introduced into the flow cell. At this point flow was stopped. When the microscope was focused on the inner face of the coverslip, fluorescent filaments were noted to bind to the surface and move forward. Movement could be recorded in a single preparation for >1 hr. At intervals, fresh buffer was infused into the flow cell to replace depleted ATP. Video records of the movement were analyzed by using an interactive video analysis program on the IBM personal computer PC/XT (17). This program divides the video screen into ≈60,000 pixels (8-bit resolution horizontally and vertically). The final magnification resulted in a video screen representing 90 μm horizontally and 70 μm vertically. Only continuous movements longer than 2.5 μm were scored to reduce quantization errors. The position of either the front or back end of the filament was recorded at a sufficient number of points to describe the path adequately.

For experiments involving EM, grids were attached to coverslips in the following manner. Formvar (0.25%) in $CHCl_3$ was applied to glass slides and allowed to dry, and the film was floated on distilled water. EM grids were placed on the film, dried and cleaned coverslips were placed over the EM grids, and the films were lifted with filter paper (Whatman no. 1). The coverslips were dried, carbon-sputter-coated, and glow-discharged. To prepare the grid for EM, it was removed from the coverslip, stained with 1% uranyl acetate, and dried with filter paper.

RESULTS

We have constructed an *in vitro* movement system in which purified single actin filaments labeled with fluorescent phalloidin are observed to move on myosin filaments fixed to a glass surface. These actin filaments exhibited ATP-dependent movement over the myosin-coated surface for distances from 2 to 50 μm. Filaments followed winding paths without reversing their direction of movement with respect to their long axis, apparently progressing from one thick filament to another. After binding to the myosin-coated surface, some filaments broke into fragments, each of which could continue to move, but often in different directions (Fig. 1). Moving

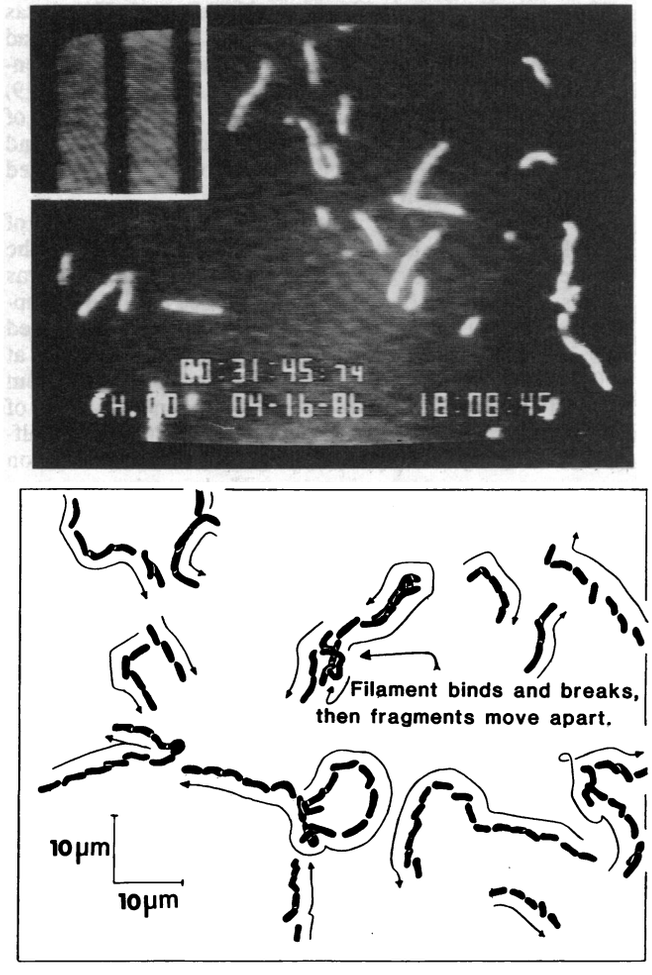

FIG. 1. (*Upper*) Photograph of a single frame of a video record of Me_4RP-labeled actin filaments observed by fluorescence microscopy. These filaments are bound to the bottom surface of a glass coverslip. (*Inset*) Photograph of a video image of the scale of the objective micrometer used for calibration of the video screen dimensions. (Divisions = 10 μm.) (*Lower*) Paths of movement of fluorescent actin filaments on a myosin-coated surface. Fourteen filaments moved during 38 s of an experiment with skeletal-muscle actin and skeletal-muscle myosin in standard buffer with 15 mM 2-mercaptoethanol. Their positions are indicated at successive short intervals as they appeared on the video monitor. The paths of the filaments and filament fragments are indicated by the curving arrows. In this experiment, the average rate of movement was ≈3 μm/s. Filaments move forward without reversing direction with respect to their long axis. Filament fragments maintain the polarity of movement of the intact filament.

filaments eventually either detach from the surface or come to a stop, bound to the surface.

We have begun to characterize the effects of a variety of factors that influence this myosin-based movement. As is true for actin-activated heavy meromyosin ATPase activity (18) and for movement in the *Nitella*-based system (19), the movement was sensitive to increasing ionic strength. Although movement was not affected by removing KCl from the standard buffer, some inhibition of movement was discernible when the KCl concentration was increased to 50 mM. Movement was substantially inhibited by 75 mM KCl and completely stopped in 100 mM KCl. $MgCl_2$ was necessary for movement at concentrations greater than the concentration of ATP, as we found for the *Nitella*-based system (19). $CaCl_2$ was not required for movement. Although the presence of reducing agent was not required, 2-mercaptoethanol may stabilize myosin and reduce photobleaching of the fluorescence.

The movement occurred over a wide range of pH and was not sensitive to the particular buffer used (Fig. 2). The broad pH optimum was somewhat different from the pH dependence of myosin movement in the *Nitella*-based system (19) but resembled the pH dependence of actin activation of myosin ATPase (20). At pH 6.5 and below and pH 9.5 and above, filaments were observed to bind to the myosin-coated surface but failed to move.

The rate of movement depended on the concentration of ATP (Fig. 3). In the absence of ATP, filaments bound to the surface and did not move. As the concentration of ATP was increased, the speed of movement increased until the asymptotic velocity at saturating ATP concentration was reached near 100 μM ATP. The half-maximal velocity occurred at ≈50 μM ATP. Although these results are quite different from the ATP concentration dependence of actin activation of heavy meromyosin ATPase activity, which shows half-maximal activity at 6 μM ATP (21), the ATP concentration dependence of movement in this purified protein movement assay is similar to that of the *Nitella*-based assay (19).

To examine the distribution of the myosin on the coverslip, we applied the myosin filaments to coverslips with EM grids attached and observed movement of actin filaments on the grids in standard buffer. When the grids were removed, stained with uranyl acetate, and observed by EM, the surface of the carbon-coated film was covered with scattered thick filaments. When the concentration of the myosin applied to the coverslips was lowered considerably (25 μg/ml or less), only an occasional actin filament bound to the surface. These filaments tended to break less frequently than those on a dense myosin substratum, and the distance of movements was one filament length or less. These results are consistent with movement of a single actin filament along a single myosin filament. These observations suggest that movement of actin over myosin requires at most the number of heads in a single thick filament.

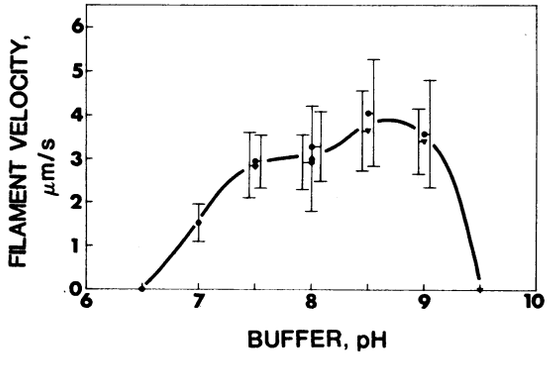

FIG. 2. The speed of skeletal-muscle actin moving on skeletal-muscle myosin depends on pH but not on the type of buffer used. The pH of the buffer was adjusted to the indicated value before addition of 2-mercaptoethanol. ●, 10 mM imidazole; ◆, 25 mM Tris; ▼, 10 mM glycine. Each data point represents the average velocity of 20–25 different filaments from a single experiment, and the standard deviations are shown by the vertical bars.

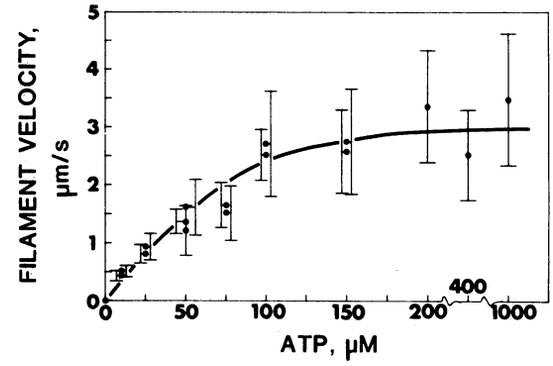

FIG. 3. The speed of skeletal-muscle actin moving on skeletal-muscle myosin depends on the concentration of ATP. Each data point represents the average velocity of 10–25 different filaments from a single experiment, and the standard deviations are shown by the vertical bars.

In previous studies using the *Nitella*-based assay (9, 19), we observed that the rates of movement depended on the type of myosin used, but the effects of different types of actin on the rate of movement could not be determined. Fig. 4 shows the results of an experiment to determine whether the rate of actin–myosin movement is controlled by the type of actin or the type of myosin. *Dictyostelium* actin and myosin and skeletal-muscle actin and myosin were used in a series of experiments in standard buffer. Both skeletal-muscle actin and *Dictyostelium* actin moved considerably slower on *Dictyostelium* myosin than on skeletal-muscle myosin. The rates of movement of the two types of actin were similar for each type of myosin. Thus, the major determinant of the rate of movement of actin and myosin is the type of myosin. It may be that actin's role in the actin–myosin interaction is passive—actin may simply serve as a catalyst for myosin movement activity.

DISCUSSION

The movement system described here has several features that make it an attractive and simple movement assay for *in vitro* studies of actin–myosin interactions. The most important feature of this assay is that it gives rapid, quantitative, and reproducible myosin movement data from small samples of purified proteins. This movement does not depend on the presence of accessory factors other than the fluorescent phalloidin used to stabilize and visualize the actin filaments. Hence, this assay offers the opportunity to examine the direct effects of modifications of either actin or myosin or of added factors on the movement process.

While the advantages of this new *in vitro* movement assay over the biochemically undefined *Nitella*-based assay are clear, various other methods have been described that have demonstrated movement in systems consisting of purified actin and myosin or its subfragments (1–6). The specific advantages offered by the system described here are (*i*) that the movement is confined to a plane, which eliminates the possibility of parallax error in measuring rates; (*ii*) that it does not require unusual buffer conditions, such as those necessary to observe movement in superprecipitation experiments; and (*iii*) that the molecules themselves are observed to move, rather than the bulk medium or attached objects.

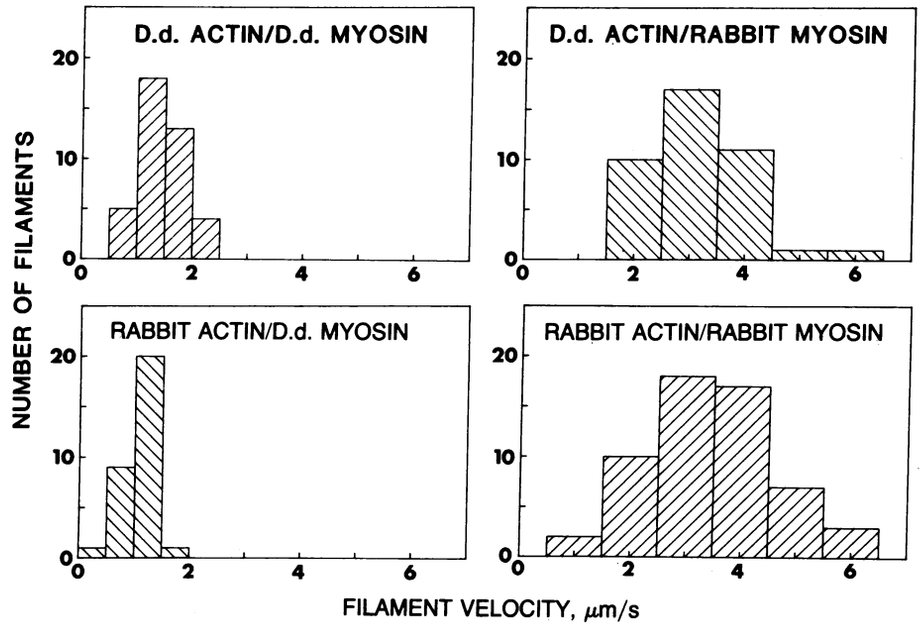

FIG. 4. Histograms comparing the rates of movement of rabbit skeletal-muscle actin (rabbit actin) and *Dictyostelium* actin (D.d. actin) on rabbit skeletal-muscle myosin (rabbit myosin) and *Dictyostelium* myosin (D.d. myosin). The average speed of *Dictyostelium* actin moving on *Dictyostelium* myosin was 1.4 μm/s (SD = ±0.4 μm/s, n = 40) and on skeletal-muscle myosin was 3.2 μm/s (SD = ±1.0 μm/s, n = 40). The average speed of skeletal-muscle actin moving on *Dictyostelium* myosin was 1.1 μm/s (SD = ±0.3 μm/s, n = 30) and on skeletal-muscle myosin was 3.5 μm/s (SD = ±1.1 μm/s, n = 57).

Our analysis of the movement of myosin-coated beads in the *Nitella*-based assay (7) suggested that the rate of bead movement could not be limited by a balance between motive force due to myosin and viscous drag on the moving beads. We found that the power output of a single myosin molecule could drive a 1-μm-diameter bead through buffer at 4 μm/s. The drag on a moving 10-nm-diameter filament is not readily calculated but is certainly much less than that on a 1-μm sphere. As with the myosin beads, the speed of the actin filaments is not limited by viscous forces. Rather, the rate of movement of the actin filaments is probably kinetically limited.

The nature of the kinetic limit on the movement of the myosin-coated beads and the single actin filaments has not been elucidated. A model consistent with our results and with the wealth of information now available on the actin–myosin system is that this kinetic limitation resides in the time needed for myosin to undergo a conformational change that results in a step of movement. This conformational change in the myosin head is likely to be the force-producing step associated with actin binding. It should be noted that because the step that kinetically limits movement is only a small part of the ATPase cycle of the myosin molecule, the changes in the rate of movement of actin filaments in this assay under different sets of conditions need not scale directly with the changes in the V_{max} of the actin-activated myosin ATPase.

As seen in the histograms in Fig. 4 and in the standard deviations in Figs. 2 and 3, the rates of movement of individual filaments have a relatively broad distribution. The scatter in the rates of individual filaments in this assay is larger than that of individual bead movements in the *Nitella*-based assay. Nonetheless, when the rates of 10 or more filament movements are averaged, the rate of filament movement is highly reproducible, and small differences in the rate of movement as a function of ATP concentration or pH are easily resolved.

A more important limitation of this assay is that, unlike rate of movement, myosin-force production is not readily measured. Theoretically, force–velocity relationships could be determined by increasing the viscous load on the moving filaments. However, as discussed above, viscous forces do not affect filament velocity in the conditions used for the experiments described here, and the magnitude of viscosity increases that are necessary to affect movement may not be practical to produce.

The movement of actin filaments on a myosin-coated substratum is in some respects analogous to the linear movement of microtubules on a glass surface in the presence of kinesin (22). In these experiments, kinesin molecules most likely attach to the glass and then can generate a translocating force on the microtubule. This suggests that, as with the case of actin and myosin, the movement producing interaction between kinesin and the microtubule is given direction by the polarity of the microtubule rather than by the orientation of the kinesin molecules.

We hope to use this system along with methods of molecular genetics to identify those aspects of actin and myosin structure that are important for coupling ATP hydrolysis to movement and to localize the regions of mobility in the myosin molecule responsible for movement and force generation. To this end, we plan to use site-directed mutagenesis of the cloned genes for *Dictyostelium* actin (23) and myosin (24) to generate modified proteins by expression in an appropriate host cell. These expressed proteins can then be characterized by a variety of techniques, including this *in vitro* movement assay.

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