ATP-dependent Movement of Myosin In Vitro: Characterization of a Quantitative Assay

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ABSTRACT Sheetz and Spudich (1983, *Nature (Lond.)*, 303:31–35) showed that ATP-dependent movement of myosin along actin filaments can be measured in vitro using myosin-coated beads and oriented actin cables from *Nitella*. To establish this in vitro movement as a quantitative assay and to understand better the basis for the movement, we have defined the factors that affect the myosin-bead velocity. Beads coated with skeletal muscle myosin move at a rate of 2–6 μm/s, depending on the myosin preparation. This velocity is independent of myosin concentration on the bead surface for concentrations above a critical value (~20 μg myosin/2.5 × 10^9 beads of 1 μm in diameter). Movement is optimal between pH 6.8 and 7.5, at KCl concentrations <70 mM, at ATP concentrations >0.1 mM, and at Mg^{2+} concentrations between 2 and 6 mM. From the temperature dependence of bead velocity, we calculate activation energies of 90 kJ/mol below 22°C and 40 kJ/mol above 22°C. Different myosin species move at their own characteristic velocities, and these velocities are proportional to their actin-activated ATPase activities. Further, the velocities of beads coated with smooth or skeletal muscle myosin correlate well with the known in vivo rates of myosin movement along actin filaments in these muscles. This in vitro assay, therefore, provides a rapid, reproducible method for quantitating the ATP-dependent movement of myosin molecules on actin.

Movement of myosin on actin filaments is believed to drive many cellular motile processes. According to the model of H. E. Huxley (9), myosin converts the energy of ATP into mechanical energy through a conformational change while it is bound to actin. Furthermore, it is generally believed that each myosin molecule acts as an independent force generator (8) and that many molecules, when coupled and acting asynchronously, provide steady movement along polar actin filaments. Until recently, actual displacement of myosin relative to actin had only been quantitated in the muscle sarcomere. The development of an in vitro assay, using myosin-coated beads to follow the position of the myosin on oriented actin cables from *Nitella* (22), now allows measurement of the rate of movement of various types of myosin on actin filaments under controlled ionic conditions.

From our previous calculations (22), we expected that the myosin-bead velocity would be similar to the maximum velocity of contraction of the muscle from which the myosin was derived. Because the actin-activated ATPase activities of myosins generally correlate with the respective muscle contraction velocities, we expected a further correspondence between bead velocity and actin-activated ATPase activity. In this study we show that these correlations do indeed hold.

The oriented polar actin cables used in the in vitro assay are derived from a dissected *Nitella* cell. Thus, the actin substratum, aside from its high degree of spatial organization (10, 11), is not well defined. It was important, therefore, to define the limits of salt concentrations and other parameters that will support myosin motility along the *Nitella* substratum. These limits may reflect in part the limits of stability of the *Nitella* substratum or the actin cables per se, or relate to the presence of auxiliary proteins on the actin cables. In fact, however, the optimal conditions in our system are consistent with the previously described (1, 7, 26) optimal conditions for the interaction of purified actin and myosin using actin-activated ATPase as an assay, which suggests that the assay is not complicated by the possible presence of auxiliary proteins on the *Nitella* substratum.

The results presented in this paper establish that the *Nitella*-based in vitro motility system is a fast, reliable, and quantitative assay for the velocity of myosin movement along actin filaments.

MATERIALS AND METHODS

Materials: *Nitella axillaris* was cultured (16) from original stocks provided by Dr. L. Taiz (University of California, Santa Cruz) and Dr. P. Richmond (University of the Pacific). In this protocol, 40 ml of chow (a mixture of 16 parts loam, 5 parts leaf mold, 6 parts fine sand, 4 parts steer manure, and

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0.02 parts bone meal, by volume), 15 ml of potting soil, and 60 ml H_2O were sterilized and then diluted in a 6 × 18 in cylindrical tank filled with distilled H_2O. After waiting for 2 d to allow particles time to settle, several terminal internodal cells from a stock *Nitella* culture were added to the tank and it was placed under 100 foot-candles of fluorescent (blue-green) illumination. Cultures normally took 2–4 wk to mature.

Skeletal myosin was prepared from rabbit muscle by the method of Kielly and Harrington (12), and was stored as a stock solution in 0.6 M KCl, 50 mM potassium phosphate, pH 6.5, 0.5 mM dithiothreitol (DTT)[1], 0.5 mM EDTA, on ice. *N*-ethylmaleimide (NEM)-inactivated myosin was prepared by reacting myosin in storage buffer with excess NEM (1 mM) for 24 h on ice. The reaction was stopped by adding 2 mM DTT. In addition, skeletal myosin samples were kindly donated by Dr. S. Lowey (Brandeis University) and Dr. R. Cooke (University of California, San Francisco). *Dictyostelium discoideum* myosin was prepared as described previously (17) with modifications detailed elsewhere (Griffith, L. M., and J. A. Spudich, manuscript in preparation). Turkey gizzard myosin, with >95% light chain phosphorylation, was a gift of Dr. J. Sellers (National Institutes of Health).

Myosin-Bead Preparation: In our standard assay, Covaspheres MX particles (1 μm in diameter; 2.5×10^{10} beads/ml; Covalent Technology Corp., Ann Arbor, MI) were diluted 10-fold with 10 mM potassium phosphate, pH 7.0, 0.2 mM DTT, and enough of a concentrated stock solution of myosin (5–10 mg/ml) to give the desired final protein concentration. In all cases, except for the myosin concentration dependence study, 100–200 μg/ml myosin was incubated with the beads. The final buffer conditions were 10 mM potassium phosphate, pH 7.0, 50 mM KCl, 0.2 mM DTT, and 0.03 mM EDTA. Beads were allowed to incubate with myosin on ice for at least 1 h before they were assayed for motility. Most often the beads aggregate into distinct groups ~5–10 μm in diameter, but the rates of movement are independent of bead diameter between 0.6 and 120 μm (22, 23). While it was possible to disrupt bead aggregates by mild sonication without affecting motility, generally this was not done.

Nitella Dissection: A *Nitella* internodal cell (2–4 cm in length and ~1 mm in diameter) was trimmed free of branch cells. The cell was rinsed briefly with distilled H_2O and placed in 10 ml of dissection buffer (10 mM imidazole, pH 7.0, 25 mM KCl, 4 mM $MgCl_2$, 4 mM EGTA, 10 mM sucrose, and 1 mM ATP). The dissection was performed at 22°C in 50-mm plastic petri dishes on a layer (2–3 mm thick) of Sylgard (Dow Corning Corp., Midland, MI). First, a cell was secured at both ends with pins of tungsten wire (length, 1–3 mm; diameter, 0.003 in) sharpened at one end by electrolysis in 10 M NaOH. Next, the cell was opened with a transverse cut using microscissors (Moria, MC19B; from Fine Science Tools Ltd., North Vancouver, B.C., Canada). The cell was then cut open along its whole length taking care to disturb as few chloroplasts as possible in the process. Finally, another transverse cut was made at each end of the lengthwise cut, and the central portion was opened and pinned flat. The cytoplasm was largely washed away in the process of cutting and pinning the cell substratum to the Sylgard. Occasionally, vesicular materials from the *Nitella* continued to move along the chloroplast rows after the cell was cut open, but this material ran off the ends of the pinned substratum within a few minutes.

Standard Assay: Myosin-coated beads were mixed 1:3 with dissection buffer containing 0.2 M sucrose and 1 mM DTT. Samples were then drawn into a microcapillary and 0.1–1 μl was applied to a limited region of the dissected *Nitella*. The assay was performed at 22°C, except in the temperature dependence study. In the figures, each data point represents the average of 5–15 beads or distinct bead aggregates.

Bead movement was recorded with a video camera mounted on a Zeiss photomicroscope and connected to a Panasonic video cassette recorder (Model NV-8050). A 40× water-immersion objective lens was used; the magnification on the monitor screen was 2,000. Our initial experiments used fluorescein-labeled beads to visualize the movement (22). The beads are easily seen, however, using bright-field optics, and this simpler method of monitoring the beads is now standard. Analysis of movement was performed on replay from the video cassette recorder where it was possible to freeze the motion. The positions of actively moving beads were traced on a piece of plastic placed on the monitor screen; the tape was then advanced and the new positions of the beads were recorded. Because the time is also recorded on the tape, the velocities of the beads were easily determined.

RESULTS

When Covaspheres coated with skeletal myosin were expelled from the microcapillary, they exhibited Brownian motion as they settled onto the *Nitella* substratum. Within 1–2 min, many beads and bead aggregates became attached to the chloroplast rows, ceased their Brownian motion, and began to move unidirectionally along the rows. As reported earlier (22), the direction of movement reversed across each of the two *Nitella* indifferent zones, consistent with the reverse in polarity of actin filaments on either side of these zones (11). The direction of movement was from the pointed ends of the actin filaments toward their barbed ends, as described previously (22). Myosin-bead movement proceeded over long distances (>100 μm) and at a constant rate independent of the size of the bead aggregate. Over time, often within 20–30 min after dissection, bead movement became less consistent: it sometimes appeared as if the beads were caught by and then broke free from invisible restraints, and some beads stopped moving altogether. The same *Nitella* could be used for measurements for ~1 h after dissection, until few enough beads were still moving smoothly and continuously for the data to be useful.

The rates of movement of beads coated with a fresh myosin preparation were highly reproducible (standard error was ~10% when 10 bead velocities were measured). These rates were consistent from one *N. axillaris* cell to another. Different skeletal muscle myosin preparations, however, moved at considerably different rates, between 2 to 6 μm/s. Furthermore, we observed that over a period of weeks, beads coated with myosin from the same preparation moved at progressively slower rates. This effect was reduced by the addition of DTT to the myosin storage buffer, suggesting that oxidation was responsible for the rate decrease. To more directly test the requirement for active myosin, we determined the effect of NEM-inactivated myosin on bead motility when mixed with active myosin. The presence of NEM-myosin dramatically slowed bead movement. When only 20% of the myosin on the beads was NEM-inactivated, movement was slowed by 50%. Therefore, it is likely that the variability of myosin-bead velocity found in beads coated with myosin from different preparations or from a single preparation over time is due to partial inactivation of the myosin.

With the bead concentration in our standard assay (where beads were diluted 10-fold to a concentration of 2.5×10^9 beads/ml; see Materials and Methods), bead velocity was constant as long as the myosin concentration in the bead

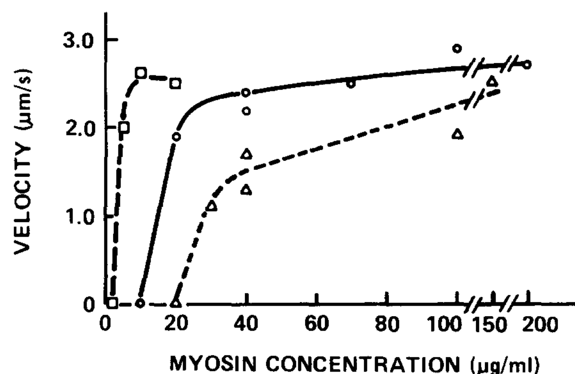

FIGURE 1 The velocity of movement of skeletal muscle myosin-coated beads is plotted versus the concentration of myosin in the bead incubation mixture for bead concentrations of (□) 6×10^8 beads/ml, (○) 2.5×10^9 beads/ml (standard assay), and (△) 1×10^{10} beads/ml. Each point represents the average velocity of five to fifteen distinct beads or bead aggregates and standard assay conditions were used (see Materials and Methods).

[1] *Abbreviations used in this paper*: DTT, dithiothreitol; HMM, heavy meromyosin; NEM, *N*-ethylmaleimide.

incubation was at least 20 μg/ml. This result was true for smooth muscle myosin and *Dictyostelium* myosin (data not shown) as well as for skeletal myosin (Fig. 1). Below 20 μg/ml there was a sharp decrease in bead velocity, and no movement at all was observed when beads were incubated with <10 μg/ml of myosin in the standard assay. The minimum concentration of myosin required for movement was found to depend on bead concentration (Fig. 1). When one-fourth the normal amount of beads was used, incubation with myosin at 5 μg/ml caused the beads to move. Conversely, when four times as many beads were used, the myosin concentration required for movement was 30 μg/ml.

We tested the relative rates of movement of myosin from skeletal muscle, smooth muscle and a nonmuscle cell (Fig. 2). The greatest velocity was observed with skeletal muscle myosin. The data shown in Fig. 2 derive from skeletal muscle myosin preparations that moved from 3 to 6 μm/s. As mentioned above, different preparations moved at different rates, but all preparations moved between 2 and 6 μm/s. Phosphorylated gizzard smooth muscle myosin, in contrast, moved at only 0.1–0.6 μm/s. *Dictyostelium* myosin moved at 0.5–1.5 μm/s. These rates of movement are thus characteristic of the type of myosin used. In addition, the rates of movement of the different myosins correlate with their relative actin-activated ATPase activities (2, 17).

The high reproducibility of the velocity measurements show that this assay is clearly a useful tool for investigating factors affecting myosin motility. To find the optimal assay conditions (defined as giving the maximum velocities) and to define boundary conditions outside of which the assay does not work, we varied several experimental parameters. As shown in Fig. 3, there is a sharp decrease in velocity below pH 6.8; above pH 7.5, there is a more gradual decline. From pH 6.8 to pH 7.5, velocity is relatively constant. Imidazole buffer and Tris-HCl buffer gave the same results. We also investigated the effect of the $MgCl_2$ concentration in the dissection buffer (Fig. 4). At 1 mM $MgCl_2$, no movement occurred. This may be because ATP, an effective chelator of Mg^{2+}, was present at 1 mM as well, and free Mg^{2+} may be required for movement. Bead velocity was maximal at 4 mM $MgCl_2$ and then gradually declined at concentrations of up to 20 mM, the highest tested.

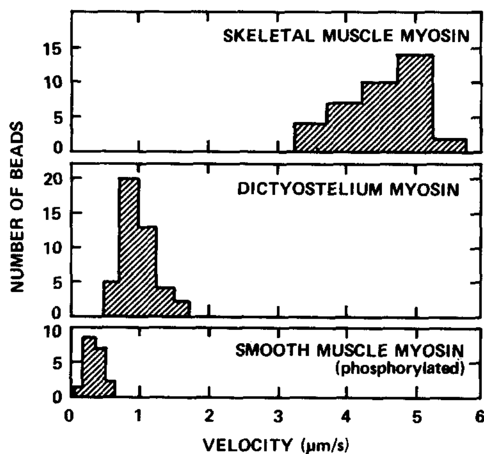

FIGURE 2 Histograms of the number of beads and bead aggregates versus bead velocity show the differences in velocity distributions for skeletal muscle, *Dictyostelium discoideum*, and phosphorylated gizzard myosin-coated beads. In all three cases 100–200 μg/ml myosin were mixed with 2.5 × 10^9 beads/ml and the standard assay was used at 22°C.

A third dissection buffer parameter we explored was KCl concentration. Dissection is usually performed in 25 mM KCl, but normal movement occurs in KCl concentrations as low as 10 mM or as high as 70 mM. At KCl concentrations of 70–100 mM, myosin-beads sometimes moved. The variability at these KCl concentrations could reflect effects on the *Nitella* substratum that differ somewhat from cell to cell. At 150 mM KCl and above no movement was observed.

Myosin-bead movement on actin cables was also found to depend on ATP concentration (Fig. 5). The velocity was half-maximal at an ATP concentration of 50 μM. The final parameter tested was the temperature at which the assay is performed. As Fig. 6 shows, velocity was found to be strongly temperature-dependent. When graphed as an Arrhenius plot the slope indicates activation energies of 90 kJ/mol from 10 to 22°C and 40 kJ/mol from 23 to 39°C.

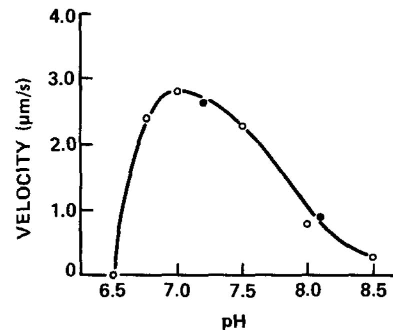

FIGURE 3 Bead velocity is plotted versus pH of the dissection medium with 10 mM imidazole (○) or Tris-HCl buffer (●) for skeletal muscle myosin using the standard assay.

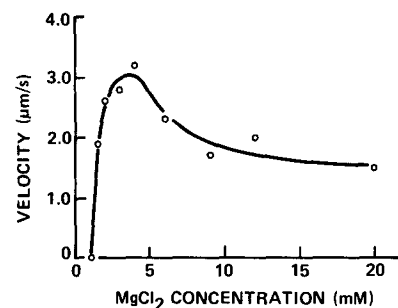

FIGURE 4 Bead velocity is plotted versus $MgCl_2$ concentration in the dissection buffer for skeletal muscle myosin.

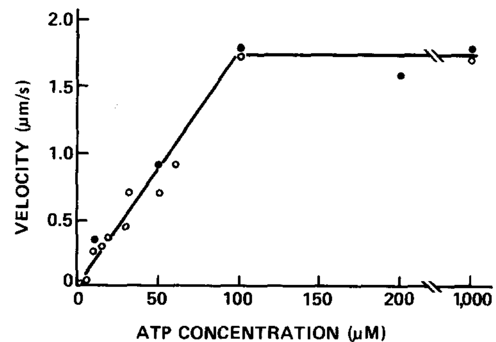

FIGURE 5 Bead velocity is plotted versus ATP concentration for skeletal muscle myosin (● and ○ denote two different myosin preparations).

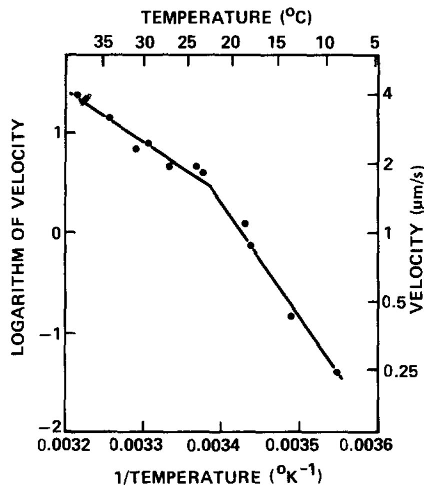

FIGURE 6 The logarithm of the velocity is plotted versus the inverse of the temperature for skeletal muscle myosin. Temperature was controlled by circulating temperature-regulated water through coils surrounding the microscope objective and within the dissection chamber. The temperature was measured at the time of the experiment by placing a thermocouple adjacent to the *Nitella*.

DISCUSSION

Myosin-bead movement on *Nitella* actin provides a quantitative assay for the fundamental function of the myosin molecule. In most cells, the rates of the relevant movements are not easily determined by direct microscopic examination. This in vitro assay allows the direct visual measurement of movement under defined buffer conditions with purified myosins. The assay is simple and fast and gives reproducible values for myosin velocity. In addition, microgram levels of myosin are sufficient to assay its movement. We normally deposit ~10 ng of myosin onto the *Nitella* substratum, but even smaller quantities can be reliably assayed.

Skeletal muscle myosin beads move at a velocity that is notably similar to the maximal relative rate of movement of myosin and actin in the muscle sarcomere (3, 6, 19). The maximal rate of rabbit skeletal muscle contraction at 25°C is about five lengths per second which corresponds to a relative movement of myosin along actin filaments of ~6 μm/s (3). Rabbit skeletal myosin-coated beads in our in vitro assay move at 2–6 μm/s at 22°C. Furthermore, smooth muscle myosin-beads move at one-tenth that velocity, in keeping with the 10-fold slower rate of contraction of smooth muscle (5, 21). These results suggest that the assay is a reliable measure of the maximal rate of cellular movements that are actin- and myosin-based.

The major weakness of the assay is that the *Nitella* substratum is not biochemically defined. There could be trace amounts of *Nitella* enzymes or proteins that modify the myosin and affect its motility. However, we have no results to date to suggest that this complication is a serious one. The cytoplasmic contents of the *Nitella* cell are diluted 2,000–4,000-fold in the dissection buffer, which significantly reduces the probability of enzymatic modification during the course of the assay. Another weakness of the assay is that *Nitella* actin could differ in important ways from other actins. For example, Nothnagel et al. (20) suggest that *Nitella* actin does not bind DNase I. However, others (Vale, R., A. G. Szent-Györgyi, and M. P. Sheetz, manuscript submitted for publication) have been able to confer calcium dependence on skeletal myosin movement by adding the tropomyosin-troponin complex from rabbit skeletal muscle to the *Nitella* actin cables. This result indicates that the *Nitella* actin does not differ significantly from other actins, in keeping with the known highly conserved nature of this protein throughout evolution (13, 27). We have recently shown that highly purified reconstituted, oriented actin filaments support movement of myosin-coated beads (24). The development of this totally defined assay will eliminate these two major concerns about the *Nitella*-based assay system.

A third problem with the assay is that the nature of the myosin on the bead surface is not well defined, and further studies are underway to clarify what configuration is required to support movement. Since the assay is carried out in 25 mM KCl, some thick filament formation, even at low myosin concentrations, probably occurs. We have not ruled out the possibility that some form of aggregation such as that found in thick filaments may be necessary. Movement of the myosin-coated beads is quantitatively similar to the bead movement reported earlier (22) using heavy meromyosin (HMM) preparations. However, in experiments using HMM preparations, only a small number of beads were moving at any one time; they moved shorter distances; and it took much longer to observe a given number of moving beads than in experiments using myosin. We wish to emphasize that although our initial observations of bead movement used HMM preparations, those preparations were not purified free from all traces of myosin. As reported (22), we estimated from SDS acrylamide gels that myosin represented ~1% of the protein in the preparations used. Numerous experiments have suggested that it is unlikely that this level of contamination could account for the movement that we have seen repeatedly with HMM preparations made as described in the Sheetz and Spudich report (22). However, all experiments to obtain movement with highly purified HMM have failed, for reasons that are not yet apparent. Thus, the critical question as to whether HMM free of any trace of myosin can move must await further experimentation.

There are good correlations between the velocity of muscle shortening and of actin-activated myosin ATPase activity (2). With regard to ATP concentration dependence, however, the half-maximal velocity of muscle shortening under zero load occurs at ~200 μM ATP (4), whereas the half-maximal value for actin-activated HMM ATPase in vitro occurs at 6 μM (18). In the case of the ATPase measurement, each myosin is expected to behave independently, so that a half-maximal rate of hydrolysis should be a reflection of 50% of the myosin ATP-binding sites being filled. On the other hand, in the case of the measurement of the velocity of shortening, the myosin molecules are not behaving independently. A small percentage of the molecules lacking ATP in their active sites would be expected to result in very strong actin-myosin interactions (rigor bonds), which should exert a substantial drag on the relative movement of thick and thin filaments. Thus, considerably more than 50% saturation of the ATP-binding sites may be required for half-maximal velocity of shortening. Our myosin-bead assay would be expected to mimic muscle shortening in this regard. The value we obtained for half-maximal velocity of movement of skeletal myosin-coated beads was 50 μM ATP, which, like the value for muscle shortening, is considerably higher than the ATP concentration required for half-maximal ATPase activity.

With regard to temperature dependence, the activation

energy measured for the velocity of shortening of intact muscle fibers is 60 kJ/mol myosin (25) and that measured for the actin-activated HMM ATPase is 120 kJ/mol (18). Interestingly, the temperature dependence of the myosin-bead velocity proved to be biphasic. This biphasic nature of the Arrhenius plot may be revealing an important property of the myosin molecule. Levy et al. (14, 15) showed nearly identical temperature dependence for actin-activated myosin ATPase activity under certain conditions. They calculated energies of activation of 50 kJ/mol >16°C and 104 kJ/mol <16°C, whereas our values for myosin movement in vitro are 40 kJ/mol and 90 kJ/mol with an inflection point at ~22°C. Levy et al. (14, 15) interpreted the biphasic character of their Arrhenius plots to reflect a reversible flexibility of the conformational state of the S1 heads of the myosin molecule. Strikingly, a very similar biphasic Arrhenius plot was obtained for the rate of decay of tension in muscle fibers by Stein et al. (25). They reported energies of activation of 70 kJ/mol >20°C and 117 kJ/mol <20°C. They interpreted the biphasic nature of the temperature dependence as reflecting a rate limiting step in the sequestering of calcium ion by the sarcoplasmic reticulum. It seems likely from the studies of Levy et al. (14, 15) that this phenomenon is an important property of the myosin molecule itself, and this conclusion is supported by our findings, where calcium ion sequestration is not an issue.

From these studies it is evident that the rapid movement of myosin past actin in the sarcomere, as well as presumably in nonmuscle cells such as *Dictyostelium*, can be reproduced in vitro with myosin-coated beads and *Nitella* actin cables, and easily measured by means of light microscopy. The rate of bead movement depends on the source of myosin, and correlates with the in vivo rates of muscle contraction as well as with the corresponding actin-activated ATPase activities. As defined here, the *Nitella* assay allows a quantitative way to study the effects of various biochemical conditions or modifications of the myosin molecule on the ability of myosin to convert chemical energy into movement.

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