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Direct Observation of Molecular Motility by Light Microscopy

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We used video-fluorescence microscopy to directly observe the sliding movement of single fluorescently labeled actin filaments along myosin fixed on a glass surface. Single actin filaments labeled with phalloidin-tetramethyl-rhodamine, which stabilizes the filament structure of actin, could be seen very clearly and continuously for at least 60 min in O_2-free solution, and the sensitivity was high enough to see very short actin filaments less than 40 nm long that contained less than eight dye molecules. The actin filaments were observed to move along double-headed and, similarly, single-headed myosin filaments on which the density of the heads varied widely in the presence of ATP, showing that the cooperative interaction between the two heads of the myosin molecule is not essential to produce the sliding movement. The velocity of actin filament independent of filament length (>1 μm) was almost unchanged until the density of myosin heads along the thick filament was decreased from six heads/14.3 nm to 1 head/34 nm. This result suggests that five to ten heads are sufficient to support the maximum sliding velocity of actin filaments (5 μm/s) under unloaded conditions. In order for five to ten myosin heads to achieve the observed maximum velocity, the sliding distance of actin filaments during one ATP cycle must be more than 60 nm.

Key words: myosin, actin, filament structure

INTRODUCTION

The purpose of this study is to observe directly molecular motility in the actomyosin energy transduction system by optical microscopy and find the minimum functional unit. The resolution of an optical microscope is restricted by the wave length used and is 0.15 μm at best. Although this resolution is too low to observe the shape of a protein molecule, the fluorescent images of molecules labeled with fluorescent dyes can be seen as bright spots using a powerful light source and a high-sensitivity detector. The fluorescent images enable us to study their translational and rotational motions, and the process of their assembly and disassembly. We have demonstrated that motions of single actin filaments labeled with phalloidin-tetramethyl-rhodamine (PHDTMR), which stabilizes the filament structure of actin, are clearly and continuously observed by fluorescence microscopy. We found that the actin filament is flexible and its bending motion becomes faster and larger in its amplitude during interaction with soluble myosin fragments, HMM or S-1, in the presence of ATP, but directional movement of actin filaments was not produced (Yanagida et al., 1984). Recently, Honda et al. (1986) and Kron and Spudich (1986) observed the sliding movement of fluorescently labeled single actin filaments along myosin filaments by fluorescence microscopy. Using these methods, we first examined the fundamental problem of whether the cooperative interaction between the two heads of the myosin molecule is required for muscle contraction. This problem has been previously studied by using actomyosin threads (Cooke and Franks, 1978), superprecipitation (Tokiwa and Morales, 1971; Margossian and Lowey, 1973; Gadasi et al., 1974), and chemical modification of the muscle fiber (Chaen et al., 1986). However, no clear conclusion has been reached because all are indirect assays. Our second interest was in the minimum number of myosin heads required for the sliding movement of actin fila-

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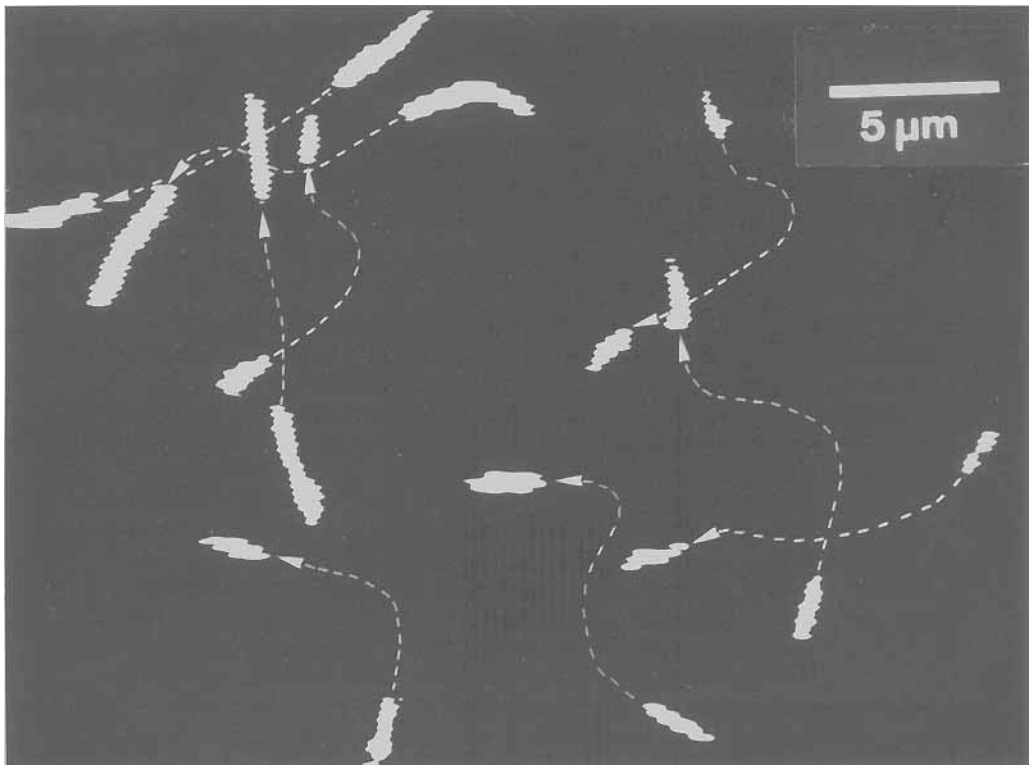

Fig. 1. Sliding movement of single actin filaments labeled with phalloidin-tetramethyl-rhodamine on a coverslip coated with single-headed myosin filaments. To demonstrate the movement of the actin filaments, two video images taken 1.5 sec apart were photographed on the same frame of a film by double exposure.

ments. Knowing this value would provide important information concerning the upper limit of the length of the sliding distance of an actin filament during one ATP cycle, a distance that may be as large as 60 nm or more (Yanagida et al., 1985) and also on whether cooperative interactions among myosin heads through an actin filament are required for the sliding of actin on myosin.

MATERIALS AND METHODS

A glass slide was coated with myosin filaments by cleaning it in EtOH then exposing it to Buffer-1 (50 mM KCl, 10 mM HEPES, pH 7.0, 1 mM $MgCl_2$, and 1 mM DTT) containing myosin filaments (Kron and Spudich, 1986). Observation by dark-field microscopy showed that for the fast movement of actin filaments, the surface of the coverslip had to be thinly and continuously covered with myosin filaments. To prepare such a surface, the concentration of myosin filaments and the incubation time were varied from 2 to 0.5 mg/ml and from 1 to 30 min at 0°C, respectively, while monitoring the surface by dark-field microscopy (Nagashima, 1986). The unbound filaments were washed away with Buffer-2 (10 mM HEPES, pH 7.0, 5mM $MgCl_2$, 0.2 mM $CaCl_2$, 1% 2-mercapto-EtOH, and 2 mg/ml BSA); then the slide was exposed to fluorescently labeled actin filaments (0.5 μg/ml) suspended in test solution (50 mM KCl, 20 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 3 mM ATP, and 1% 2-mercapto-EtOH). The movements of the actin filaments were observed at 23°C (Fig. 1). Actin was obtained from rabbit skeletal muscle and purified by the method of Spudich and Watt (1971). Actin filaments were labeled with phalloidin-tetramethyl-rhodamine by a method described previously (Yanagida et al., 1984).

Single-headed myosin filaments were prepared by two methods (Fig. 2 A). In the first preparation (SHM-1), myosin filaments were digested by papain (Margossian and Lowey, 1973) until double-headed myosin was barely detectable by Coomassie-blue–stained polyacrylamide gels, and single-headed myosin was purified by the methods of Cooke and Franks (Cooke and Franks, 1978). Short, thick filaments (~0.6 μm long) were formed by rapidly suspending in Buffer-1 the purified preparation of single-headed myosin, dissolved in a high ionic solution (Huxley, 1963). The purity was checked by 3% polyacrylamide gel electrophoresis in the presence of 20 mM PPi (Cooke and Franks, 1978). The gel

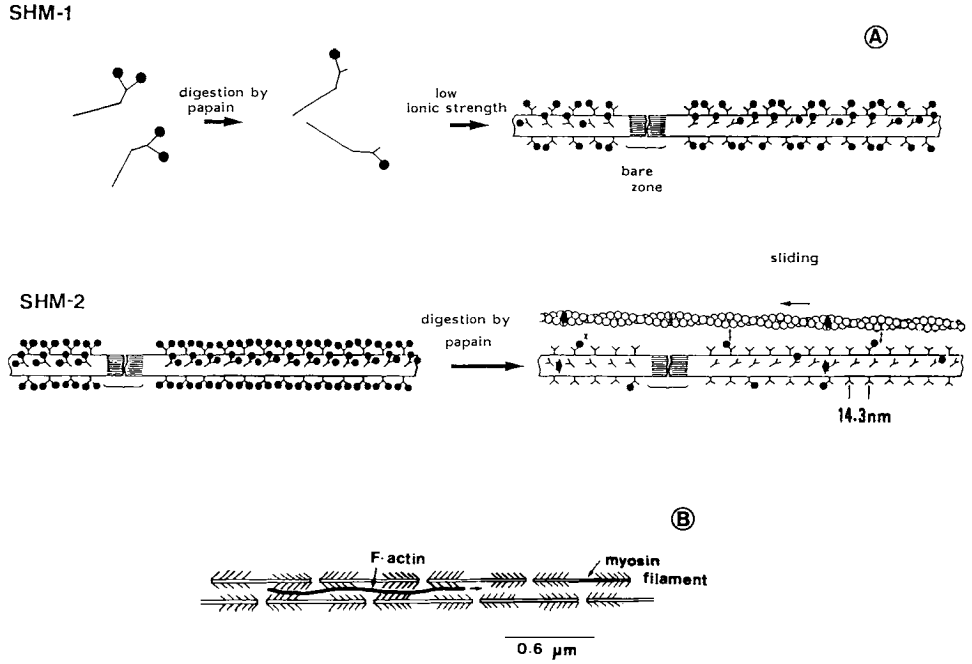

Fig. 2. (A) Preparation of single-headed myosin filaments. (B) Arrangement of myosin filaments on a glass surface assumed for calculation of number of myosin heads which interact with moving F-actin.

patterns of typical samples show the sample contained 91% single-headed myosin, 8% rods, and 1% or less double-headed myosin (on a molar basis). The concentrations of the myosin species determined by the present gel method have been shown to agree reasonably with those found by electron microscopy (Cooke and Franks, 1978). Myosin was obtained from rabbit skeletal muscle and purified by a method described previously (Yanagida et al., 1984). In the second preparation (SHM-2), more than 90% of the myosin heads were removed by papain digestion from synthetic myosin filaments, which had been formed by rapid dilution from 0.6 M KCl. The digested heads were removed by centrifugation, and the treated filaments were used without further treatment. The gel of a typical sample (SHM-2) had 15% single-headed myosin and 85% rods, in a molar ratio. The line corresponding to double-headed myosin was hardly seen on the gel even if the sample was overloaded; its content, therefore, was probably less than 0.5%.

Single fluorescent actin filaments were observed with an inverted microscope (Zeiss IM35) equipped with epifluorescence optics, a Zeiss Neofluoar x100 object (oil immersion, NA, 1.3), a 100 W mercury arc lamp and a Zeiss rhodamine filter (excitation = 554 nm; emission >580 nm). Their images were recorded on videotape with a high sensitivity television camera (Ikegami SIT and ISIT camera CTC-9000) and a video recorder (Victor CR 6650L). The fluorescence images were enhanced in their contrast and analyzed with an image process (TVIP-2000 Avionics Co., Japan) as needed.

RESULTS AND DISCUSSION

Sensitivity of the Fluorescence Microscope System

The fluorescence intensity from actin filaments labeled with PHDTMR excited by a 100 W ultra-high pressure mercury arc lamp was approximately equal to that using a 20 mW Ar Laser (514 nm) with a 40 μm beam diameter. Therefore, the photon density provided by the mercury arc lamp is assumed to correspond to that of the Ar Laser, 10^{16} photons (514 nm)/sec/(10^3 μm^2). Taking into account the absorption coefficient and quantum yield of tetramethyl-rhodamine and the numerical aperture of the objective lens, the number of photons that are emitted from a single tetramethyl-rhodamine molecule and reach the photoelectric surface of SIT Camera is estimated to be 10^5 photons/sec. If the size of the fluorescent image of a single dye molecule is assumed to be (0.01 mm^2) on the photoelectric surface of SIT camera, the density of photons on the photoelectric surface is calculated to be 10^7 photons/sec/mm^2, which is much larger than the density required for observation by the SIT camera, 10^5 photons/sec/mm^2. This indicates that a single dye molecule emits a sufficient number of photons to be seen by the present fluorescence microscope system. An actin filament 0.5 μm long that is

bound to 100 PHDTMR molecules gave fluorescence image too bright to be clearly seen on the TV monitor screen under maximum illumination, supporting this estimation. Recently, we have shown that actin filaments less than 40 nm long that have less than eight PHDTMR molecules can be seen in O_2-free solution by the present system (Harada and Yanagida, in preparation).

Movement of Actin Filaments on Myosin Filaments

Fluorescently labeled actin filaments were added to a glass slide coated with myosin filaments and the movements of actin filaments were observed directly by fluorescence microscopy. The most rapid movement of actin filaments along double-headed myosin filaments was observed in a buffer containing 50 mM KCl and 3 mM ATP. The time averaged velocity was 7.2 μm/sec but the instantaneous velocity exceeded 10 μm/sec. This is about 2-fold faster than that during maximum shortening of muscle fiber, but is expected to be the maximum velocity under truly unloaded conditions because such fast shortening was seen in single sarcomeres after removal of Z-line and connective tissue (Yanagida et al., 1985). When the concentration of KCl was decreased from 50 to 0 mM, their average velocity decreased from 7.2 to 5.1 μm/sec at 23°C, which agreed with the results obtained for chemically skinned muscle fibers (Thames et al., 1974). At more than 75 mM KCl, the number of moving actin filaments greatly decreased, and the sliding movement was no longer observed at KCl concentrations greater than 100 mM.

Sliding Movement of Actin Filaments on Single-headed-Myosin Filaments

The sliding velocity of actin filaments along thick filaments formed from purified single-headed myosin (SHM-1) or double-headed myosin (DHM) was similar in the range of 0 to 30 mM KCl. At over 30 mM KCl, few actin filaments interacted with the thick SHM-1 filaments in the presence of 3 mM ATP. When the concentration of ATP was decreased to 100 μM, however, sliding movement was observed even at 75 mM KCl. The preparations of single-headed myosin used in this study contained less than 1% double-headed myosin on a molar basis. Therefore, to test the possibility that the contaminating double-headed myosin might be responsible for the movement of actin filaments, we observed the movement of actin filaments on glass coated with myosin filaments that were prepared in one of three ways as follows: 1) rods without heads, obtained by papain digestion of myosin, and double-headed myosin were mixed in a molar ratio of 99:1 and the hybrid filaments, formed when the ionic strength decreased, were added to the coverslip; 2) a 99:1 mixture of rods and intact myosin in high-ionic medium were brought into contact with the coverslip and then filaments were allowed to form on the surface; and 3) a mixture of filaments consisting of rods (99% molar ratio) and double-headed myosin (1%) that had been separately formed was brought into contact with the coverslip. In all cases, no movement of actin filaments was observed, indicating that the contaminating double-headed myosin was not responsible for the movement. Furthermore, as mentioned below, actin movement was observed in a mixture of single-headed myosin and rods, although there was less than 0.5% (SHM-2) of a contaminating double headed-myosin. These results suggest that the double-headed structure of myosin is useful for increasing the affinity of myosin for actin, but a cooperative interaction between the two heads is not essential for the development of sliding force. The recent finding by Yano-Toyoshima et al. (1987) that myosin subfragment-1 and single-headed HMM can produce the sliding movement of actin filaments supported our result.

This result, however, does not allow us to exclude the possibility that the original cooperative interaction between the two heads of myosin might be replaced by interactions between two adjacent single heads, since the density of the heads on the thick filaments was very high. To examine this possibility we measured the sliding movement of actin filaments along thick filaments which had a low density of single heads. These surfaces were prepared by thinning more than 90% of the heads by papain digestion. The gel pattern showed that the treated filaments contained 85% rods and 15% single-headed myosin, in a molar ratio. The line corresponding to double-headed myosin could hardly be seen; its content was probably less than 0.5%. In the range of 0 to 15 mM KCl, the actin filaments were observed to move along such filaments at almost the same velocity as that before thinning of the heads. This almost completely excludes the possibility of pairing of adjacent single heads on the filament, because the average interval between adjacent heads amounts to 34 nm as shown below. When the content of single-headed myosin was decreased to less than 15%, both the velocity of actin filaments and the number of moving filaments decreased and at less than 5%, moving filaments were no longer observed. This result agrees with the above one that the mixture of double-headed myosin and rods, in a molar ratio of 1:99, could not maintain the movement of actin filaments. These results support the conclusion that a cooperative interaction between the two heads of myosin is not essential for inducing the sliding movement of actin filaments (Harada et al., 1987).

The second point we studied was how many heads are required for the sliding movement. The data using SHM-2 showed that the sliding velocity of actin fila-

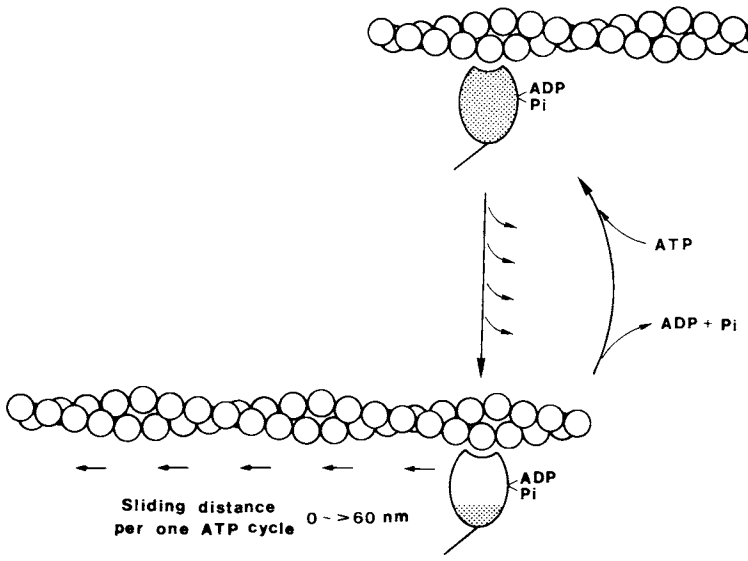

Fig. 3. Free energy of ATP is stored in a myosin head in the form of M-ADP-Pi and a predominant chemical state is AM-ADP-Pi during generation of sliding force (Nagano and Yanagida, 1984). The stored energy, which is shown by small dots for convenience' sake, is usually not used at once during a single power stroke cycle but is taken out in small quantities that are used during many power stroke cycles. The number of power strokes during one ATP cycle depends on the load. Therefore, an actin filament moves along the myosin head by from 0 to more than 60 nm depending on the load during one ATP cycle. When the actin filament can rotate freely around its long axis, the actin filament moves along the myosin head, rotating for the binding sites to orient toward the myosin head in turn. When the actin filament cannot rotate, for example, in skeletal muscle, the energized head can not complete continuously all power stroke cycles, and therefore the head performs a few cycles only while the binding sites of actin filament orient toward it and repeats discontinuously such cycles many times during the shortening. Since many heads interact with an actin filament in muscle, while one head does not interact with the actin filament other heads produce the sliding force. Thus, in the actomyosin energy transduction system, the chemical reaction is not coupled in a one-to-one fashion with the mechanical reaction, and the conversion from chemical energy to mechanical energy is flexibly performed depending on the conditions.

ments was hardly changed until 93% of heads were removed from the thick filaments. We used this to estimate the minimum number of heads required to achieve the maximum velocity. If we assume that the synthetic filament contains six heads (three myosin molecules) per 14.3 nm, like intact thick filaments, then the average interval between adjacent single heads along the filament would be about 34 nm (six myosin heads $\times$ 0.07 / 14.3 nm = one single-headed myosin/34 nm) (Fig. 2 A). Although thick filaments are not well ordered on the cover slip, let us consider the case in which an actin filament 1 μm long moves along a rail on which such single thick filaments are connected in series. One half of the heads on the thick filament bound to the glass surface is thought to be oriented toward the actin filament. A thick filament 0.6 μm long should have heads available for interaction with the actin in one third of the region, because the heads on one side of the filaments, for which the polarity is the opposite of that of the actin filaments, do not interact with actin, and the bare zone (0.2 μm) has no heads. The average number of myosin heads available for interaction with this running actin filament 1 μm long can be calculated to be 1 μm $\times$ 1 head/34 nm $\times$ 1/2 $\times$ 1/3 = 5 heads. It is possible that actin filaments move between two thick filaments in a staggered array, in which case the number would be 5 $\times$ 2 = 10 heads (Fig. 2 B). Observations of myosin filaments on the coverslip by dark-field optical microscope showed that an actin filament is unlikely to move a long distance (>5 μm), while sandwiched upward and downward by two pairs of myosin filaments in a staggered array. The actin filament is not likely to interact with more thick filaments at the same time. Another possible factor causing an increase of this number arises from the fact that the actin filament might move to the place where myosin heads are densely localized by chance. However, this possibility would be unlikely because 1) many short actin filaments, e.g., less than 1 μm long, moved continuously over a long distance (>5 μm) at the maximum velocity and homogeneously on the whole surface of the coverslip; 2) it is unlikely that thick filaments on which heads densely remain due to uneven digestion are by chance connected in series, with long rails (>5 μm) formed by such filaments are homogeneously spread on the whole surface of the cover slip; and 3) inertia is negligible for the movement of actin filaments (Yanagida et al., 1984). Thus, the results suggest that five, or at most ten heads are sufficient to

support the movement of actin filaments at the maximum velocity.

We estimated the sliding distance of an actin filament during one ATP cycle using this number. The turnover rate for myosin during sliding cannot be directly detected. Recently, however, Honda et al. (1986) have shown that the sliding movement occurred at about 5 μm/sec (27 °C) during the interaction in solution, indicating that the ATPase rate obtained in solution is not very different from that obtained during the in vitro assay. Therefore, we used the V_{max} obtained using the actomyosin solution under the same conditions, which was about eight ATP/head/sec (data not shown), as the turnover rate for myosin in this assay (Honda et al., 1986). The sliding distance of actin filaments during a single ATP cycle is calculated as 5 $\mu m/sec^{-1}/5(5-10$ heads $\times$ 8 ATP cycles $head^{-1}$ $sec^{-1}) = 130-60$ nm/ATP cycle, assuming that the ATP cycles occur in turn without overlapping. The actual value should be larger because the ATP cycles sometimes occur simultaneously. The ATPase rate during the sliding may be higher than the V_{max}, but since the sliding velocity along regularly arrayed myosin filaments as assumed here should be about 2-fold faster than the time-averaged velocity (5μ/sec) as mentioned above, this factor would be canceled. Thus, the sliding distance is probably 130 nm or more, and must be at least 60 nm under unloaded conditions. This point recently has been supported in a more convincing way. We found that the sliding velocity of actin filaments on monomeric myosin fixed on the surface of silicone-treated coverslip is independent of the filament length over a wide range from 40 nm to more than 10 μm (Harada and Yanagida, in preparation). The number of myosin molecules that can interact with an actin filament 40 nm long is probably only one, but no more than two. In order for an average of one or two myosin molecules to achieve the observed maximum velocity (3 to 6 μm/sec), the sliding distance of actin filaments during a single ATP cycle must be larger than 60 nm (Fig. 3).

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