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Stepwise Motion of an Actin Filament over a Small Number of Heavy Meromyosin Molecules Is Revealed in an *In Vitro* Motility Assay[1]

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In order to determine the relative motions of an actin filament and a myosin molecule upon hydrolysis of one ATP, an *in vitro* motility assay, in which individual actin filaments slide over heavy meromyosin molecules bound to a substrate, was combined with an optical trapping technique. An actin filament, attached to a gelsolin-coated bead, was captured with an optical trap. The surface-bound heavy meromyosin molecules pulled the filament against the trapping force, which resulted in back and forth motions of the actin-bound bead. The number of heavy meromyosin molecules interacting with an actin filament (at most 1/μm filament) and the ATP concentration ($\leq$0.5 μM) were chosen so as to facilitate detection of each "pull." Calculation of the centroid of the bead image revealed abrupt displacements of the actin filament. The frequency of such displacements was between 0.05 and 0.1 per 1 s per 1 μm actin filament, being consistent with calculated values based on the reported bimolecular binding constants of ATP and the actomyosin rigor complex. The distribution of the displacements peaked around 7 nm at a trapping force of 0.016 pN/nm, but it became broader, and some displacements were as large as 30 nm, when the trapping force was reduced to 0.0063 pN/nm, suggesting that the force generation due to the structural change of a myosin head may be insufficient to explain such displacements.

Key words: actomyosin force, motor protein, optical trapping.

In muscle contraction, relative sliding between actin and myosin occurs upon hydrolysis of ATP by myosin. The molecular event underlying this process is the displacement of an actin filament by one myosin molecule upon hydrolysis of an ATP molecule by the myosin head. In this report we term this process the elementary event. Many models have been proposed [for example, Refs. *1-4*] to explain the elementary event, but because they were derived through experiments involving muscle fibers and actomyosin solutions, both containing large numbers of actin and myosin molecules, these models should be tested at the level of a single molecule. The *in vitro* motility assay can fulfill such a requirement, because the number of HMM molecules supporting the sliding of individual actin filaments and the ATP concentration can be varied over wide ranges (*5, 6*). Thus, by lowering both the number density of HMM and the ATP concentration in this assay, one can expect to detect the occurrence of the elementary event. To measure the movement and the force, we prepared "bead-tailed" actin filaments (Suzuki *et al.*, manuscript in preparation) and manipulated the filament by trapping the actin-bound bead with an optical trap (*7*). The trapping of the bead reduced its random motion, thereby facilitating determination of its displacement from the trap center (*8*).

HMM was prepared from rabbit skeletal myosin by chymotryptic digestion (*9*). Actin filaments were prepared as described (*10*). All chemicals were of analytical grade.

The motility assay (*11*) combined with the optical trapping technique (*7*) was performed as follows. HMM was adsorbed to a nitrocellulose-coated coverslip (see below). Actin filaments were visualized with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR), and then bound to gelsolin-coated polystyrene beads (0.88 μm diameter; Polyscience, Warrington, PA) by mixing the filaments and the beads at an appropriate ratio, followed by overnight incubation at 0°C. A bead carrying only one sufficiently long (between 3.5 and 4.6 μm) filament was selected under a fluorescence microscope (Nikon TMD; Nikon, Tokyo; equipped with an oil-immersion objective, 100×, NA 1.3, with a phase ring). The selected actin-bound bead was captured with a home-made optical trap apparatus (with a 350 mW Nd-YAG laser ALC1064; Amoco Laser, IL; as the light source), and was held at about 1 μm above the HMM surface during the measurements. Methylcellulose (0.2%)

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Abbreviations: CCD, charge coupled device; HMM, heavy meromyosin.

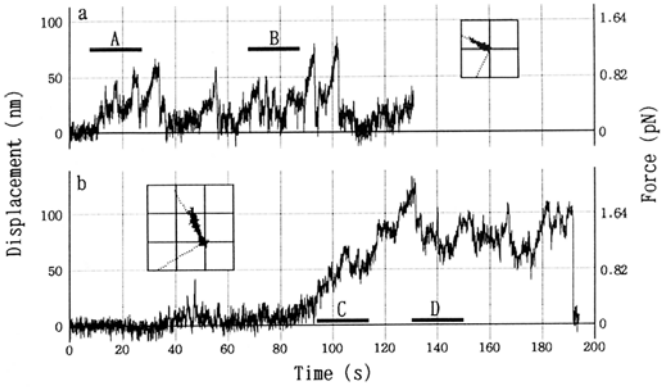

Fig. 1. **Two representative traces of the bead motion obtained at 0.25 μM (a) and 0.5 μM (b) ATP.** Horizontal bars indicate the range which is shown in Fig. 2 with an expanded time scale (A and B for Fig. 2, c and d; C and D for Fig. 2, g and h). Ordinates: left, the bead displacement; right, the trapping force. Insets: x–y traces of displacement of each bead. The major direction of the bead displacements is taken as the x direction. Grids, 100 nm.

was added to reduce dissociation of the actin filament from the HMM surface (*12*).

The fluorescence image of the bead-tailed filament and the phase-contrast image of the bead were simultaneously observed with a modified W-microscopy apparatus (*13*): the fluorescence images were taken with an image intensifier (KS1381; VIDEO SCOPE, Washington, DC) connected to a CCD camera (CCD-72; DAGE MTI, IN), and the phase-contrast images, used to calculate the bead position, with another CCD camera. The two images were combined electronically (MV24-C; For A, Tokyo) and then recorded on 8 mm video tape (EVO-9650; Sony, Tokyo) for further processing.

The bead position was determined at 33 ms intervals from the centroid of the bead image calculated with an image processor (DIPS-C2000; Hamamatsu Photonics, Shizuoka). The position of the trap center was determined from the video sequence in which the actin filament was not interacting with the HMM surface. The potential for the trapping force was estimated from the distribution of the positions of an actin-free bead in the trap at reduced laser intensities; for this measurement, we raised the viscosity of the medium to avoid image blurring due to fast Brownian motions. The potential was found to be approximated by that of a Hookean spring up to 200 nm from the trap center.

To determine the surface density of HMM on a substrate, HMM (1 μg/ml) was infused into a flow chamber (thickness $\sim$50 μm) constructed from one nitrocellulose-coated and one uncoated coverslip, and then allowed to adsorb for 1 min. The difference between the amount of infused HMM and that of unadsorbed HMM was determined from the NH_4-EDTA ATPase activity: about 80% of the infused HMM was adsorbed, giving a density of 150 HMM per μm^2.

The ATP concentration used in our experiment (<1 μM) is lower than the usual values (*5*), and hence the rate of ATP binding, which triggers the generation of force and motion, is expected to be at most 1 per 1 s per myosin head, given the bimolecular rate constant of ATP binding (*14*) of $0.2\text{–}1\times10^6\ M^{-1}\cdot s^{-1}$. Thus, individual events should be observable at a video rate of 30 Hz. The HMM density (about $150/\mu m^2$) was also low so that the elementary

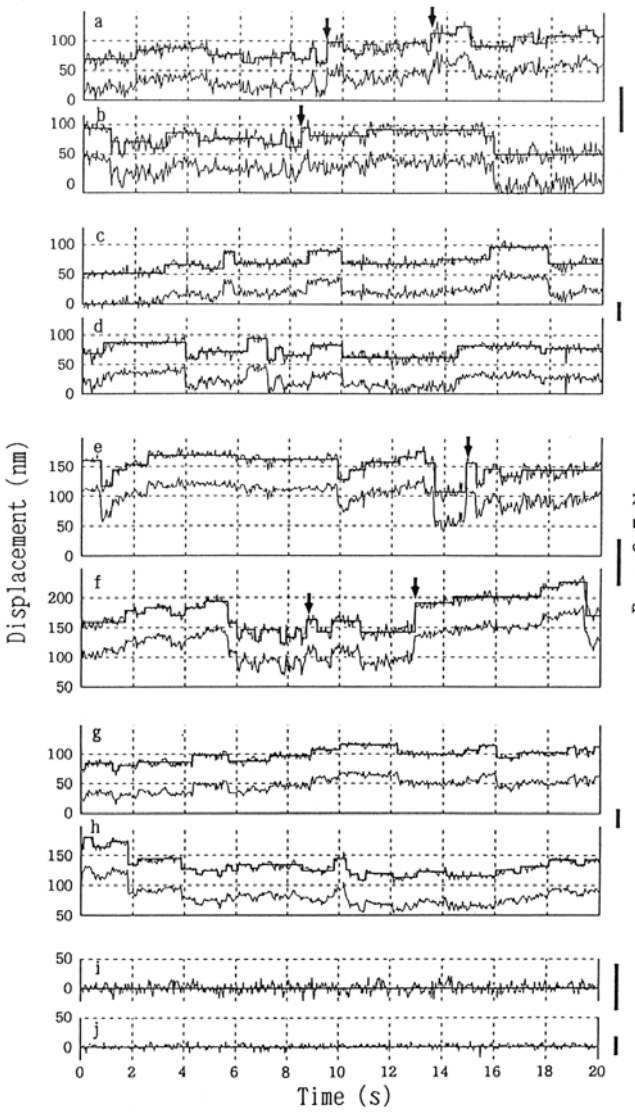

Fig. 2. **Examples of the stepwise motion of an actin-bound bead observed at 0.25 and 0.5 μM ATP.** The steps identified by eye are indicated with bold lines. Due to random noise it was not possible to count all stepwise displacements in the upper traces in each panel (a–h). a to d, the steps observed at 0.25 μM ATP; e to h, the steps at 0.5 μM ATP. a, b, e, and f, the steps at 0.0063 pN/nm; c, d, g, and h, at 0.016 pN/nm. i and j, traces obtained when the actin filament was not in contact with the HMM surface are shown for comparison. Arrows indicate where the displacements of $\gtrsim$30 nm occurred. Ordinates, bead displacement in nm. The vertical bars on the right indicate 0.5 pN.

events could be resolved, but still high enough to hold actin filaments on the HMM surface in the presence of ATP ($\leq$0.5 μM).

Bead motions in the presence of ATP consisted of forward (motion away from the trap center) and backward displacements (Fig. 1, a and b). That these motions were caused by a small number of HMM was confirmed in the absence of ATP (rigor conditions), where almost all HMM near an individual actin filament binds tightly to the filament. Fluorescence observation of the actin filaments on the HMM surface indicated that the individual filaments were bound to the surface through several points, in the order of 1 point per 1 μm filament. In another experiment,

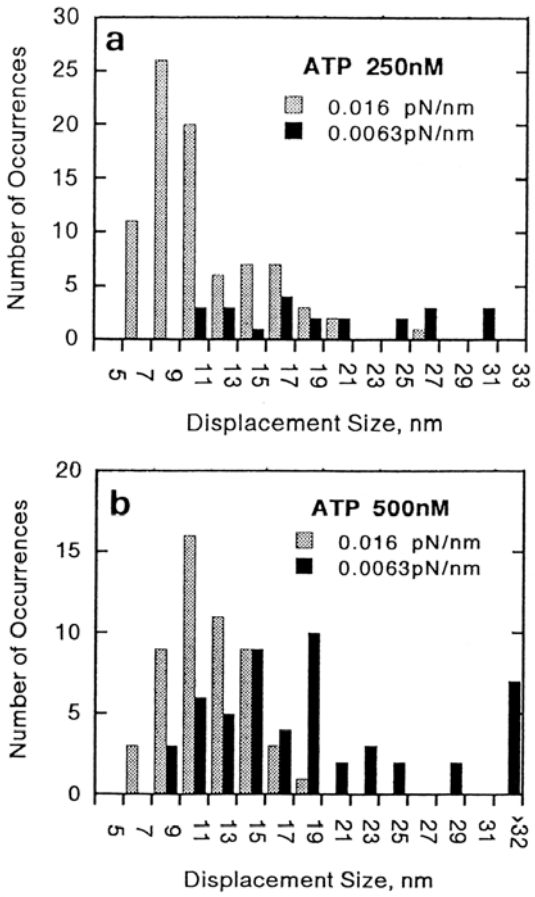

Fig. 3. **Distributions of sizes of steps determined for forward displacements.** a, at 0.25 μM ATP; b, at 0.5 μM ATP. Shaded bars, at 0.016 pN/nm; bold bars, at 0.0063 pN/nm.

a bead-tailed actin filament bound to the HMM surface was torn off by pulling the bead with the optical trap: the abrupt dissociations of the filaments from the individual HMM were counted. Hence, the number per 1 μm filament was also about 1 or less. ATP caused dissociation of some of these rigor heads, but most of the undissociated heads must have remained in the rigor state to hold the filament at the surface because of the low ATP.

Examination of the bead motion with an expanded time scale revealed abrupt displacements, which occurred within scores of milliseconds (Fig. 2, a to h). Because of the noise level (Fig. 2, i and j), we only took those displacements which had a magnitude of $\geq$5 nm as abrupt displacements. The frequency of the displacements was between 0.05 and 0.1 per 1 s per 1 μm actin filament.

Each of these displacements most likely resulted from the elementary event. The stationary bead position between the steps is expected to be determined by a balance among the three forces: (a) the force of the heads which pulls the bead away from the trap center (positive force), (b) the force of the other heads which pulls the bead backward (negative force), and (c) the trapping force. If a head exerting the negative force is dissociated by ATP binding, the force balance is disrupted and the bead advances, which is immediately followed by rebinding of the dissociated head to achieve a further advanced position. Two successive advancements will be recognized as a single displacement in most cases because of our low time resolution. If a head exerting the positive force is dissociated, on the other hand, a backward motion occurs, which is immediately followed by a forward motion as a result of reassociation and force generation. The final position in this case would be positive, negative or zero.

If the above interpretation is correct, the frequency of abrupt displacements should be equal to the rate of ATP binding; from the bimolecular rate constant (*14*) and the number of interacting HMM molecules per 1 μm actin filament, the values are calculated to be 0.05–0.5 at 0.25–0.5 μM ATP per 1 s per 1 μm filament, which are consistent with the experimental results, if one considers that we did not count the small displacements buried in the random noise.

The size distribution of the forward displacement peaked around 7 to 9 nm with a stronger trap at both ATP concentrations (Fig. 3, a and b). The rather wide distribution of the displacement size, as compared with in the case of kinesin (*8*), might be due to some difference in the mechanism of force generation between kinesin and myosin (*15*), or, perhaps because of the structural differences between microtubules and actin. Since a kinesin molecule makes 8 nm steps along a protofilament of a microtubule, this step size may reflect the size of the tubulin dimer, the structural unit of a protofilament (*8*). In the case of actin, each helical strand of an actin filament might impose a structural restriction on the binding of each myosin head to an actin monomer, so that the interval between the bound heads might not be so regular, except for the 37 nm interval arising from the periodicity of the two-start helix of an actin filament.

When the trapping force was weaker, the size distribution was broader with no clear peak, and the size of some displacements was $\gtrsim$30 nm. These displacement sizes are close to or within the recently suggested range of distances over which a head displaces an actin filament with one ATP (*12, 16*). If the displacement for this elementary event is as small as 15 nm, our displacement size of 30 nm is still explainable: two heads successively hydrolyze two ATP molecules to produce a displacement which will be twice the above distance. The probability of the occurrence of such a process must be low, but cannot be ignored in our experiments. However, a displacement of 6 nm, which has been derived from a proposed structural change of a myosin head (*17*), seems to be too small to account for our value. Thus, if structural changes in fact cause the displacements, changes in other portions of a myosin molecule might be involved. A mechanism in which a head hydrolyzes one ATP and generates the force for a longer distance at a lower load than conventionally assumed (*6*) might be another possible way to explain our value.

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