Mechanical Measurements of Single Actomyosin Motor Force

Hidetake Miyata,* Hiroshi Yoshikawa*, Hiroyuki Hakozaki,† Naoya Suzuki,§ Taiji Furuno,¶ Akira Ikegami,¶ Kazuhiko Kinosita Jr.,* Takayuki Nishizaka,‖ and Shin'ich Ishiwata‖

*Department of Physics, Faculty of Science and Technology, Keio University, Kohoku-ku, Yokohama 223; ‡Nikon Corporation, Yokohama Plant, Sakae-ku, Yokohama 244; §Department of Physics, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01; ¶Department of Physics, Faculty of Medicine, Keio University, Kohoku-ku, Yokohama 223; and ‖Department of Physics, School of Science and Engineering, Waseda University, Shinjuku-ku, Tokyo 169, Japan

ABSTRACT To elucidate the mechanism of force generation by actomyosin motor, a measuring system was constructed, in which an in vitro motility assay was combined with an optical trapping technique. An actin filament of several μm long was attached to a gelsolin-coated polystyrene bead, and was allowed to interact with a small number (~1/1-μm actin filament) of rabbit skeletal heavy meromyosin (an active subfragment of myosin) molecules bound to a nitrocellulose-coated coverglass. The bead position was determined at 33-ms intervals. We measured the force generation event at relatively low (100–400 nM) ATP concentration so that the occurrence of individual force generation events could be detected with our time resolution. The actin-bound bead held in the optical trap moved in a stepwise manner in the direction of the actin filament only in the presence of ATP. At the trap strength of 0.3 pN/nm, the maximum size of the step was 11 nm, and the maximum force associated with the movement was 3.3 pN.

INTRODUCTION

Muscle contraction is driven by cyclic interaction of two contractile proteins, actin and myosin, and this process is fueled by chemical energy of ATP, which is hydrolyzed by individual myosin heads. As one ATP is hydrolyzed by a myosin head, power stroke by the head occurs and actin filament is displaced. Recently, several groups (Finer et al., 1994; Ishijima et al., 1994; Miyata et al., 1994) have carried out experiments to elucidate the molecular mechanism underlying the process. In these experiments an in vitro motility assay was combined with a micromanipulation technique to measure the small force and the displacement; when a small number of myosin heads were allowed to interact with an actin filament that was bound to and manipulated with a microneedle or a μm-sized plastic bead, abrupt motion of the microneedle or the bead was observed in the presence of ATP. Previously, we measured the actomyosin motor force with relatively weak trapping force (trap constant = 0.0063–0.016 pN/nm) and found that the maximum force was about 0.5 pN (Miyata et al., 1994), which was significantly smaller than the value obtained by other groups (~3–5 pN; Finer et al., 1994; Ishijima et al., 1994). From our results it was suggested that the force was larger when the trap was stronger, so that we expected that use of a stronger trap would be necessary to measure larger force. In the experiments reported here we have extended our measurement using a trap stronger than the previous one (up to 0.3 pN/nm).

Address reprint requests to Dr. Hidetake Miyata, Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama-shi, 223 Japan. Tel.: 81-45-5631141 ext. 3975; Fax: 81-45-5631761

0006-3495/95/04/286s/05 $2.00

MATERIALS AND METHODS

Materials

Heavy meromyosin (HMM) and actin were prepared from rabbit skeletal muscle. Actin filaments were labeled with rhodamine-phalloidin (Molecular Probes, Inc., Eugene OR). Polystyrene beads (diameter = 0.88 μm) were from Polysciences, Inc. (Warrington, PA). All other chemicals were of analytical grade. A complex of actin filaments and gelsolin-coated polystyrene beads was prepared according to the method described by Suzuki (manuscript in preparation). The length of actin filaments was 3–10 μm.

Methods

The in vitro motility assay was carried out according to the method described by Kron et al. (1991). HMM was bound twice to a nitrocellulose-coated coverglass at 2 μg/ml for 1 min at room temperature. The number of HMM interacting with 1 μm actin filament was ~1 (Miyata et al., 1994). Relatively low ATP concentration (100–400 nM) was maintained with ATP regeneration system (100 μg/ml creatine kinase and 1 mM creatine phosphate). To help the actin filaments bind to a small number of HMM, 0.2% methyl cellulose was added (Uyeda et al., 1991).

To measure the force and motion, an optical trap was integrated into an inverted microscope (TMD, Nikon Tokyo, Japan) (Fig. 1 *a*). Laser light from an yttrium-lithium-fluoride-laser light source (1053 nm, 1 W, OEM 1053–1000p, Amoco Laser Co., Naperville, IL) was focused with an objective lens (numerical aperture = 1.3, 100×, Nikon) to form an optical trap. The trap constant was determined from the distribution of the position of a bead undergoing Brownian motion in the trap potential (for details, see Miyata et al., 1994). The bead position was determined at 33-ms intervals from calculation of the centroid of the phase contrast image of the bead. The potential was found to be axisymmetrical and approximated with that of a Hookean spring up to 200 nm from the trap center, and the trap constant was 0.05–0.3 pN/nm.

In individual experiments an actin-bound bead was held with the optical trap at ~1 μm above the glass surface, and the filament was allowed to interact with the surface-bound HMM (Fig. 1 *b*). The fluorescence image of the bead-bound filament was observed simultaneously with the bead image to ensure that the motion occurred in the filament direction, which is expected if the motion is due to the active interaction of HMM with the actin filament.

In our measurement one actin filament was bound with several myosin heads, and most of the heads were in rigor state, because ATP concentration

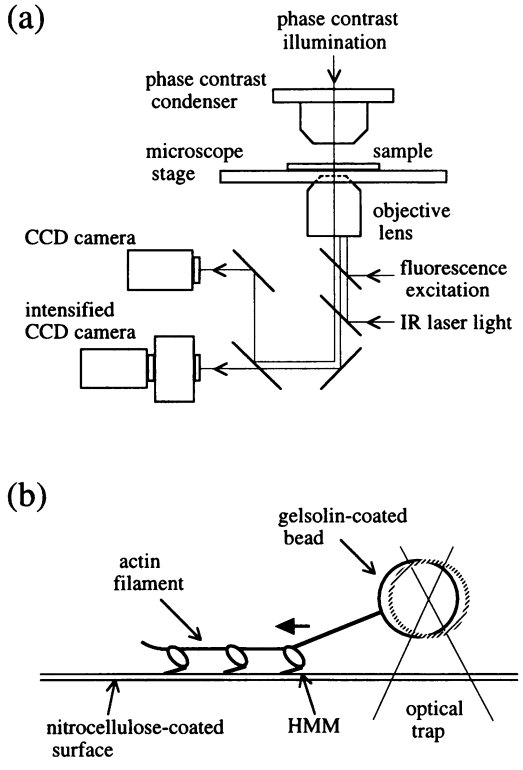

FIGURE 1 Schematic representation of the inverted microscope-based piconewton force measuring system. (*a*) A simplified diagram of the system. The phase contrast and the fluorescence image of the bead and the filament were separated with a dichroic mirror, and were received with a charge-coupled-device (CCD) and an intensified CCD camera, and were electronically combined (not shown). (*b*) A bead-bound actin filament interacting with the HMM molecules bound to a nitrocellulose-coated coverglass. The bead was held with the optical trap at about 1 μm above the surface; thus the filament was tilted when interacting with HMM. Note that the figures are not drawn to scale.

was low. Also for this reason, only one ATP molecule bound to one of those heads at a time. The binding of ATP to one of the heads caused the dissociation of the head from the actin filament, and the force generation (power stroke) immediately followed. This would move the bead: the final bead position is expected to be determined by a balance among the positive and negative force of the heads, and trap force (Miyata et al., 1994). Each event should be easily resolved at a video rate under our experimental condition, because a rate of ATP binding under our condition must be low due to the low ATP concentration, and the small number of HMM interacting with an actin filament. (The bimolecular rate constant of ATP binding to acto-S-1 in solution is $\sim 10^6$/M·s (Goldman, 1987).

RESULTS AND DISCUSSION

Stepwise motion

Fig. 2 (*a–e*) shows several examples of the bead motion (arrows), which we regard as stepwise and as a result of the force generation by individual heads of the surface-bound HMM. These traces were obtained in the presence of 100 and 200 nM ATP at the trap strength of 0.3 pN/nm. In these examples the bead position changed abruptly (within scores of ms). These motions occurred only in the direction of the filament and in the presence of ATP, indicating that the motion was caused by the active interaction between actin and HMM. In the presence of 400 nM ATP, or at different trap strength (0.05 and 0.1 pN/nm), a similar type of motion was observed (not shown).

Under our experimental conditions, binding of ATP to each head initiated the power stroke, which was expected to cause the abrupt change in the bead position. Then, the frequency of the occurrence of the abrupt motion was expected to be equal to the rate of ATP binding. The duration of the motion was on the order of a fraction of a second, which was consistent with the rate of ATP binding. This result further supports the above indication that the abrupt motion was caused by the active interaction of HMM with the actin filament.

To analyze the size of the steps, we selected the abrupt motion with the size ≥4 nm. With this criterion, the measured averaged size of the step at 200 nM ATP was 5.8 nm at the trap strength of 0.3 pN/nm, 5.4 nm at 0.1 pN/nm, and 5.3 nm at 0.05 pN/nm, respectively. Occasionally, the bead moved in a gradually ascending manner over several seconds: this type of motion can occur if many myosin heads are interacting with an actin filament and individual heads independently pull the filament for a short distance. However, we could not determine whether this was the case, and no analysis was made on this type of motion.

The magnitude of the force obtained as a product of the averaged size of the step and the trap constant was 1.7, 0.54, and 0.27 pN at each trap strength. The maximum force, 3.3 pN, was obtained at 200 nM ATP and at the trap strength of 0.3 pN/nm. In a few cases a force of about 3 pN was obtained, but in most cases the force was smaller. In our previous study the size of the step at the trap strength of 0.016 pN/nm was around 8 nm: the data shown here indicate that the size of the step showed only ~30% decrease, but the force increased by ~6 times upon increase in the trap force. It seems that it is the size of the step, rather than the magnitude of the force, that is definitive. Definitiveness of the size of the step accords with the proposed mechanism of the power stroke, in which stereospecific interaction between actin protomers and a myosin head is assumed (Rayment et al., 1993).

Effect of the tilt of the actin filament

The maximum force (3.3 pN) was smaller than that obtained by other groups (Finer et al., 1994, Ishijima et al., 1994). The force (i.e., the size of the step × the trap constant) would be smaller if the size of the step smaller than the actual size of the power stroke. We consider here three factors that can make the step smaller. (1) Tilt of the actin filament: the actin filaments stretched between the bead, and the surface-bound HMM always had some tilt (~30°, under our experimental conditions); thus, the magnitude of the bead motion caused by the displacement in horizontal direction between HMM and actin was smaller by a factor of $\cos^2\theta$, where θ is the tilt angle of the actin filament. Thus, the observed bead displacement was probably ~1.3 times smaller than the actual displacement between HMM and actin filament. (2) Slack of

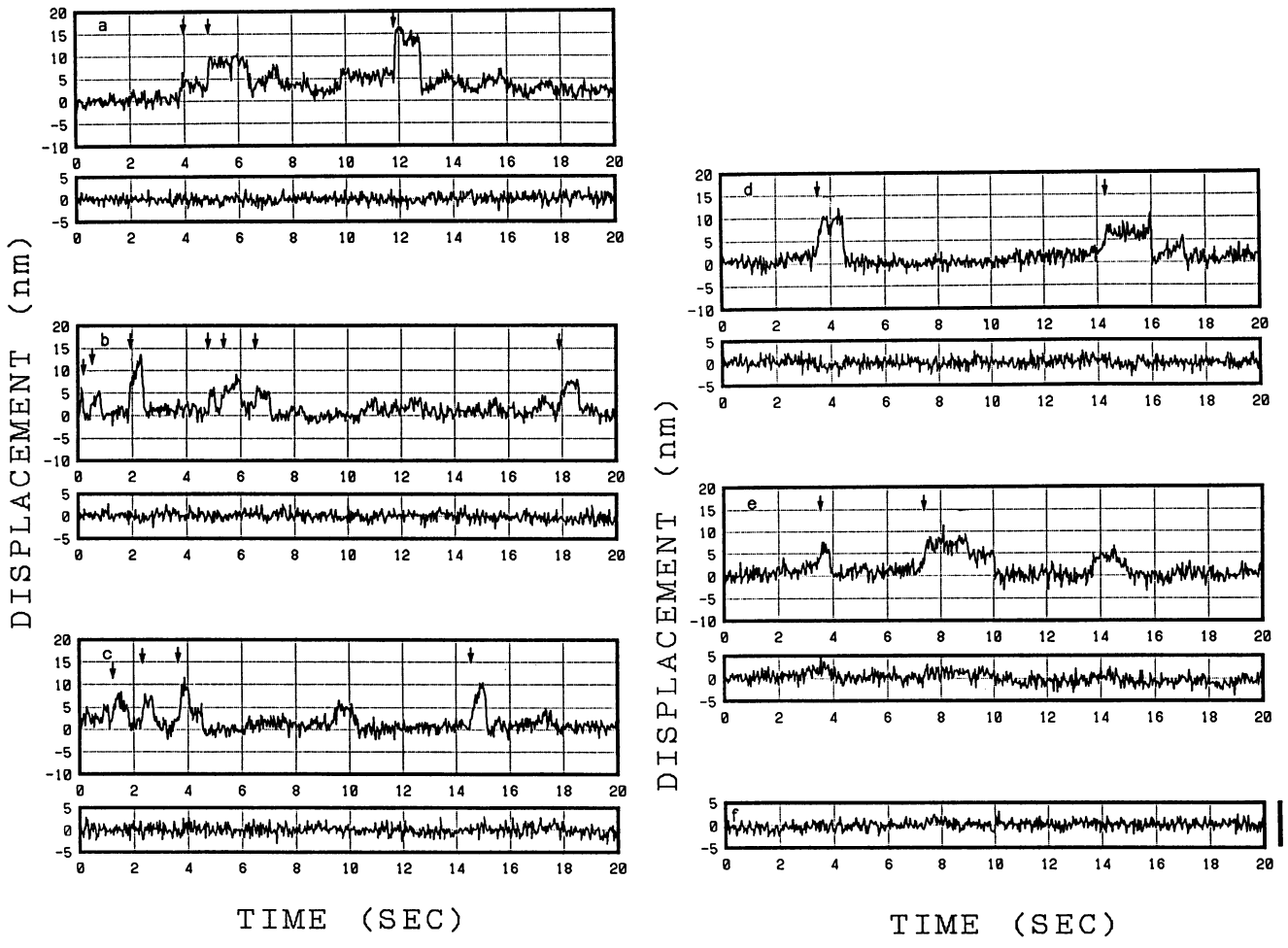

FIGURE 2 Several examples of the stepwise bead motion (indicated by arrows) measured at 0.3 pN/nm trap strength. (*a*) The traces obtained at 100 nM ATP. (*b–e*) The traces at 200 nM ATP. In each set the upper and the lower traces were the record in the direction parallel to and perpendicular to the actin filament direction. In (*e*) the filament direction changed in the middle of the measurement and the slight change in Y direction observed at the beginning almost disappeared in the later stage. (*f*) The trace obtained when the actin did not interact with the surface indicating the level of thermal noise and other types of noise (e.g., mechanical). A bar next to the trace (*f*) indicates 3 pN force.

the actin filament: it is obvious that the power stroke of HMM cannot be effectively transmitted to the bead, if the filament is slack. (3) Existence of large compliance: if large compliance exists somewhere between actin-HMM and HMM-surface linkages (Nishizaka et al., 1995), such compliance makes the bead displacement smaller (Svoboda and Block, 1994).

If we correct the maximum force for factor (1), the force will be ~4.3 pN, closer to the other groups' values, but the averaged force is still smaller: we should also consider factors (2) and (3), but the effect of these factors is more difficult to quantitatively evaluate. We qualitatively consider the effect of the latter two factors in the next section.

The effect of the slack of an actin filament and the compliance

As pointed out above, if the bead-bound actin is slack, or if a large compliance exists, the surface-bound HMM molecules cannot "effectively" pull the bead. When first bound to the HMM molecules, the actin filament itself, or the individual "springs," may not have been sufficiently stretched. (The term "spring" is used hereafter as a substitute for the term "high compliance" for the sake of convenience.) Nevertheless, we did observe the bead motion. This implies that the slack of the filament eventually disappeared, and the springs became more stretched. This seems to be obvious at first, because a small number of HMM can move an actin filament (Uyeda et al., 1991), thereby hauling the filament to achieve these tasks. However, this can only be possible when the HMM molecule nearest to the bead holds the filament throughout the process and the force generation process as well, because dissociation of this HMM would allow the filament to diffuse away and produce again the slack (Fig. 3).

In our experiments the bead motion became observable immediately to ~1 min after the contact of the actin filament with the surface-bound HMM was achieved. This may be explained by variability of the time required for the disappearance of the slack. Even after the motion became observable, the large step and hence the large force was rarely observed. We suspect that this was due to the insufficient stretch of the spring, because one actin filament al-

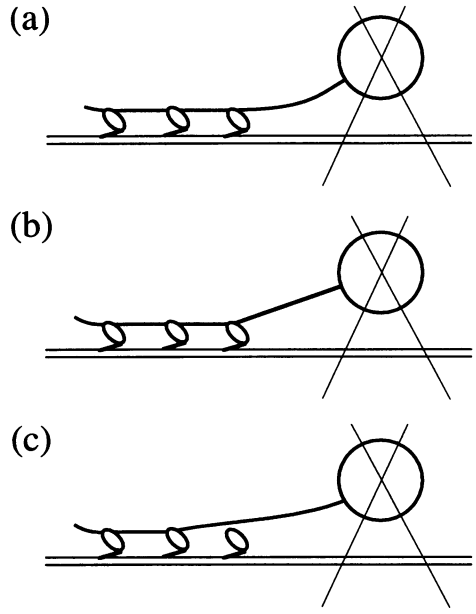

FIGURE 3 The slack of an actin filament and the bead motion. (*a*) When the actin filament (——) is slack, the power stroke of the surface-bound HMM molecules is not effectively transmitted to the bead (○). (*b*) As HMM molecules haul at the filament it eventually becomes straight and the power stroke is effectively transmitted. (*c*) If the HMM nearest to the bead dissociates from the actin, the slack again occurs.

ways interacted with several heads, and it is conceivable that not all the springs had been fully stretched.

The mechanism that enables the hauling process is not clear. Because of the low ATP concentration, it may be that one head of HMM bound the filament in a rigor state while the other head executed a power stroke, or two heads may have alternately functioned as suggested for directional movement of kinesin on a microtubule (Hackney, 1994).

Electron microscopy of HMM

To understand and explain our results in light of the structural change in HMM, we have initiated electron microscopy of HMM molecules. Details will be published elsewhere (T. Furuno, manuscript in preparation). Briefly, a negative-staining solution dissolving HMM was dried on a silicone surface by a spin-coating technique (Furuno et al., 1992), and electron micrographs of the HMM molecules were taken. By preparing the HMM sample in the above manner, we found that the detailed shape of head and tail portion of HMM was well preserved. HMM molecules with straight or slightly curved heads were observed in the absence of MgATP, whereas HMM with sharply bent heads were observed in its presence. It is not clear at the present moment whether this conformational difference in head portion is related to the observed event of force generation, but the above observation seems to be consistent with the result of the analysis of the small-angle x-ray scattering measurements, which has suggested conformational change in S-1 in solution in the presence of ATP (Wakabayashi et al., 1992), as well as the model of the conformational change in S-1 deduced for the x-ray crystallography (Rayment et al., 1993).

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan, by Special Coordination Funds for Promoting Science and Technology from the Agency of Science and Technology, and a grant from Keio University.

REFERENCES

Finer, J. T., R. T. Simmons, and J. A. Spudich. 1994. Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature.* 368: 113–119.

Furuno, T., K. M. Ulmer, and H. Sasabe. 1992. Scanning electron microscopy of negatively stained catalase on a silicone wafer. *Microsc. Res. Tech.* 21:32–38.

Goldman, Y. E. 1987. Kinetics of the actomyosin ATPase in muscle fibers. *Annu. Rev. Physiol.* 49:637–654.

Hackney, D. D. 1994. Evidence for alternating head catalysis by kinesin during microtubule stimulated ATP hydrolysis. *Proc. Natl. Acad. Sci. USA.* 91:6865–6869.

Ishijima, A., Y. Harada, H. Kojima, T. Funatsu, H. Higuchi, and T. Yanagida. 1994. Single-molecule analysis of the actomyosin motor using nano-manipulation. *Biochem. Biophys. Res. Commun.* 199:1057–1063.

Kron, S. J., Y. Y. Toyoshima, T. Q. P. Uyeda, and J. A. Spudich. 1991. Assays for actin sliding movement over myosin coated surfaces. *Methods Enzymol.* 196:399–416.

Miyata, H., H. Hakozaki, H. Yoshikawa, N. Suzuki, K. Kinosita Jr., T. Nishizaka, and S. Ishiwata. 1994. Stepwise motion of an actin filament over a small number of heavy meromyosin molecules is revealed in an *in vitro* motility assay. *J. Biochem.* 115:644–647.

Nishizaka, T., H. Miyata, H. Yoshikawa, S. Ishiwata, and K. Kinosita Jr. 1995. Mechanical properties of single protein motor of muscle studied by otpical tweezers. *Biophys. J.* 68:75s

Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, M., K. C. Holmes, and R. A. Milligan. 1993. Structure of the actin-myosin complex and its implications for muscle contraction. *Science.* 261:58–64.

Svoboda, K., and S. M. Block. 1994. Force and velocity measured for single kinesin molecules. *Cell.* 77:773–784.

Uyeda, T. Q. P., H. M. Warrick, T. J. Kron, and J. A. Spudich. 1991. Quantitized velocities at low myosin densities in an in vitro motility assay. *Nature.* 352:307–311.

Wakabayashi, K., M. Tokunaga, I. Kohno, Y. Sugimoto, T. Hamanaka, Y. Takezawa, T. Wakabayashi, and Y. Amemiya. 1992. Small-angle synchrotron X-ray scattering reveals distinct shape changes of the myosin head during hydrolysis of ATP. *Science.* 258:443–447.

DISCUSSION

Session Chairperson: Kenneth A. Johnson
Scribe: Michelle Wang

PAUL DRIEZEN: Have you looked at the possible effect that might be caused by pairwise interactions of heavy meromyosin?

HIDETAKE MIYATA: No.

DRIEZEN: Obviously that was a leading question. In a poster presented at this meeting, Umesh Ghodke has shown some evidence that confirms Harrington's original work and