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Single-Molecule Mechanics of Heavy Meromyosin and S1 Interacting with Rabbit or *Drosophila* Actins Using Optical Tweezers

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ABSTRACT Single-molecule mechanical interactions between rabbit heavy meromyosin (HMM) or subfragment 1 (S1) and rabbit actin were measured with an optical tweezers piconewton, nanometer transducer. Similar intermittent interactions were observed with HMM and S1. The mean magnitude of the single interaction isotonic displacements was 20 nm for HMM and 15 nm with S1. The mean value of the force of single-molecule interactions was 1.8 pN for HMM and 1.7 pN with S1. The stiffness of myosin S1 was determined by applying a sinusoidal length change to the thin filament and measuring the corresponding force; the mean stiffness was 0.13 pN nm^{-1}. By moving an actin filament over a long distance past an isolated S1 head, we found that cross-bridge attachment occurred preferentially at a periodicity of about 40 nm, similar to that of the actin helical repeat. Rate constants for the probability of detachment of HMM from actin were determined from histograms of the lifetime of the attached state. This gave a value of 8 s^{-1} or 0.8×10^6 M^{-1} s^{-1} for binding of ATP to the rigor complex. We conclude (1) that our HMM-actin interactions involve just one head, (2) that compliance of the cross-bridge is not in myosin subfragment 2, although we cannot say to what extent contributions arise from myosin S1 or actin, and (3) that the elemental movement can be caused by a change of shape of the S1 head, but that this would have to be much greater than the movements suggested from structural studies of S1 (Rayment et al., 1993).

INTRODUCTION

Optical tweezers (Ashkin et al., 1986) have been used previously to control position and to measure the mechanics of interaction between kinesin and microtubules (Svoboda and Block, 1993) and between myosin and actin (Finer et al., 1994). We have designed and built a transducer based upon optical tweezers as outlined in Fig. 1, similar in principle to that reported by Finer et al. (1994). In our apparatus, several beads can be suspended simultaneously by chopping a single laser beam between different positions, enabling a single actin filament to be attached to a bead at each end and held taut in a specific position and orientation.

We have performed some preliminary experiments to address the following questions:

1) Does single-headed S1 produce the same force and displacement as HMM?

2) What is the force and displacement produced by HMM interacting with actins of different structure?

3) Is the slower F-actin sliding velocity, measured in in vitro assays, of wild-type *Drosophila* and even slower mutant $ACT88F^{E93K}$ due to a foreshortened work stroke or because of lower cross-bridge force?

4) Does S1 interact preferentially with actin monomers that have favorable azimuthal orientation?

5) Is cross-bridge detachment distortion-dependent?

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Abbreviations used: BSA-TRITC, bovine serum albumin-tetrarhodamine isothiocyanate; NEM, *N*-ethyl-maleimide; HMM, heavy meromyosin; WT, wild type; SDS-PAGE, sodium dodecyl sulphate, polyacrylamide gel electrophoresis; E93K, glutamic acid to lysine at amino acid position 93.

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MATERIALS AND METHODS

Protein purification

Actin and HMM

Rabbit F-actin was prepared using a standard method (Pardee and Spudich, 1982). The flight muscle-specific isoform of actin (Act88F) from *Drosophila* was isolated by a scaled-down purification of dissected indirect flight muscles. From 10 flies, we obtained a yield of 5 μg of actin.

Rabbit myosin and chymotryptic HMM were prepared essentially as described by Margossian and Lowey (1982), with the exception that TLCK-treated, α-chymotrypsin was used and its activity stopped with Bowman-Birk inhibitor.

Production of S1

Rabbit skeletal myosin was digested under conditions of low salt and high free magnesium in the presence of activated papain (12 mg/ml myosin in 100 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, 10 mM imidazole, pH 7.3, 10 μg/ml activated papain, 22°C, 15 min). The reaction was stopped by a twofold excess of E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane). Digest products were dialyzed to low ionic strength, and precipitated material was pelleted by centrifugation. The supernatant was column purified (DE52, Whatman, Kent, U.K.), and the central peak of the eluate was concentrated by ultrafiltration (Amicon Ltd. Stonehouse, Gloucs, U.K.) in the presence of 30% sucrose. S1 aliquots were frozen to −80°C. The S1 ran as a single, symmetrical, peak on ion-exchange (DEAE, Whatman) and gel filtration (Sephacryl S200, Pharmacia, Uppsala, Sweden) chromatography. However, SDS-PAGE (gels not shown) indicated that in addition to the intact 95-kDa heavy chain (about 50% of total product), fragments of 75- and 20-kDa were also present in this preparation. All three types of light chain were present.

Production of fluorescently labeled, NEM-myosin-coated latex beads

1.1 μm latex (polystyrene) beads (Sigma Chemical Co., St. Louis, MO) were coated with BSA-TRITC (1 μg/ml) and NEM-treated monomeric myosin (10 μg/ml) (prepared as Sekine and Keilley, 1964) by incubating 0.1% beads, by mass, at 4°C for 1 h in a 0.5 M KCl, buffered (pH 7) solution.

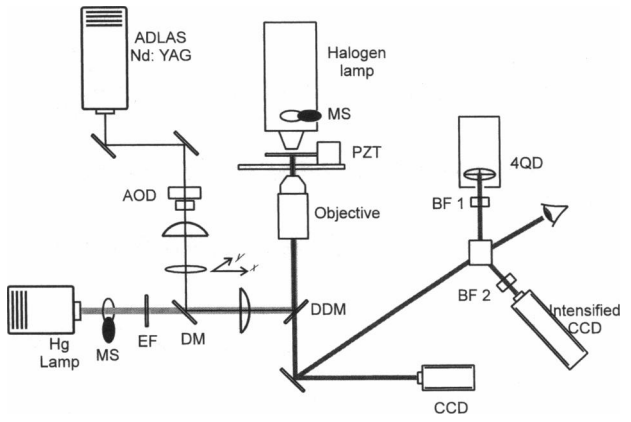

FIGURE 1 Apparatus block diagram. The optical trap is built around an inverted microscope (Axiovert 135, Zeiss, Oberkochen, Germany). Infrared (1064 nm) laser light, from a diode-pumped Nd:YAG laser (Adlas 321, Adlas, Lubeck, Germany) is combined with green light (EF, 546FS10.25, excitation filter, Andover Corp., Salem, NH) from a mercury arc lamp using a "hot mirror" (DM = 820DCSP, Omega Optical, Brattleboro, VT). Both light beams enter the microscope epifluorescence port via a custom-built housing. A dual dichroic mirror (DDM, 570DCLP, Omega Optical; reflects 546 and 1064 nm; transmits >570 nm), mounted in the microscope filter block, allows us to use optical tweezers and view rhodamine fluorescence simultaneously. Laser beam alignment is via two mirrors and control of trap position is made with two orthogonally mounted acousto-optic deflectors (AODs) (NEOS, Melbourne, FL); these are controlled by a custom-built computer interface card. To produce two optical traps, we chop between two sets of x, y coordinates (to simplify computation, these coordinates are chopped in hardware at 10 kHz). The laser light path is completely enclosed using cardboard tubing and low density foam rubber to prevent air currents from entering the system at any point. Coarse control of stage position is by mechanical drives and a custom-built piezoelectric substage (PZT) allows small range, computer-controlled movements of the microscope slide. High speed position measurements are made with a 4-quadrant photodiode (4QD, S1557, Hamamatsu Photonics, Hamamatsu City, Japan) and custom-built electronics. Scattered laser light is excluded from this detector (BF1, short-pass barrier filter). Fluorescently labeled actin is viewed with an intensified CCD camera ("Photon - P46036A", EEV, Chelmsford, U.K.) coupled to a barrier filter (BF2 = LP590, Zeiss). Bright-field illumination (100 W halogen lamp) is used to produce a high magnification video image (CCD camera, P46310, EEV). An Acroplan 100X, 2.5 N.A. objective and an Optovar 2.5x insert are used to obtain the desired image magnification. Computer-controlled mechanical shutters (MS) are used to switch between bright-field and fluorescence illumination.

Bead agglomeration was reduced by final addition of 0.5 mg/ml BSA and dispersion by brief ultrasonication at 4°C.

In vitro assays

In vitro motility assays were performed according to Kron et al. (1991). All motility assays were performed at 23°C.

Mechanical measurements made by optical tweezers

Single actin filaments were attached between two NEM-myosin-coated, 1.1-μm diameter latex beads, held in two independently controlled laser traps. The position of one of these beads was monitored by imaging the bead on a quadrant photodetector (Finer et al., 1994). HMM or S1 was supported on a 1.7-μm-diameter glass microsphere attached to a coverslip, and the interactions with the actin filament were enabled by moving the actin filament close to the HMM- or S1-coated glass microsphere. To measure forces and displacements produced by single-motor molecules, the concentration of HMM or S1 applied to the flow-cell was reduced until summation of events no longer occurred. For both S1 and HMM, about 1–2 μg protein/ml was required. This concentration is reasonable if one assumes that all myosin molecules bind to the nitrocellulose surface, and that the "reach" of an actin filament covers a 30 × 300 nm^2 area of the 1.7-μm glass microsphere. This area would then contain about 2–5 molecules of motor protein.

In free-run (quasi-isotonic) mode, the stiffness of the laser traps was 0.02 pN nm^{-1} each, giving an overall system stiffness of 0.04 pN nm^{-1}; this stiffness, much less than that of the myosin subfragments, allowed estimates of the elemental movement produced by the interactions between the myosin fragments and the actin. Thermal motion of the bead, while held in the trap, limits the minimum observable stroke size. Because trap and cross-bridge stiffnesses are in series, bead displacement produced by the cross-bridge stroke depends on the relative value of the two stiffnesses.

In feedback (force) mode, the bead was held in a fixed position, forces exerted by the molecular interactions being compensated by movement of the laser trap, allowing estimates of the isometric force of interaction between the molecules. Controlled length changes were applied by imposing movement onto the laser beam holding the bead imaged on the photodetector, the force being measured from the movement of the laser beam required to produce the correct movement of the bead. By this means, stiffness- and distortion-dependent kinetics of the myosin/actin interactions were determined.

RESULTS

Myosin structure

Comparison of responses obtained with rabbit HMM and S1

Fig. 2 shows the interactions obtained between single molecules of rabbit heavy meromyosin and S1 with rabbit actin filaments measured at 10 μM ATP. Responses are shown with the apparatus in (A) force and (B) displacement modes.

Forces seen with S1 are similar to those observed with HMM, from which we conclude that only one HMM head is producing force under the conditions we have used. The mean value of the forces seen, about 1.8 pN (SD = 0.86 pN, n = 724), is less than that, 3.5 pN, reported by Finer et al. (1994) for HMM, as are the maximum values of our 4.5 pN, compared with their 7 pN.

The mean value of displacements seen with S1 (14.8 nm; SD = 5.8; n = 264) is significantly less (Student's t-test) than seen with HMM (19.7 nm; SD = 7.8; n = 261). However, it is possible that the HMM data contain some double strokes and histograms of both data sets (not shown) indicating that the distribution is not normal. So we cannot conclude that the elemental step size of S1 is shorter than that of HMM. The important finding is that for both S1 and HMM displacements of 15 nm are frequently observed.

Actin structure

Comparison of responses seen with different actins

We have measured the force and displacements seen between actin filaments prepared from different sources and rabbit HMM. Fig. 3 shows typical force traces obtained with actin

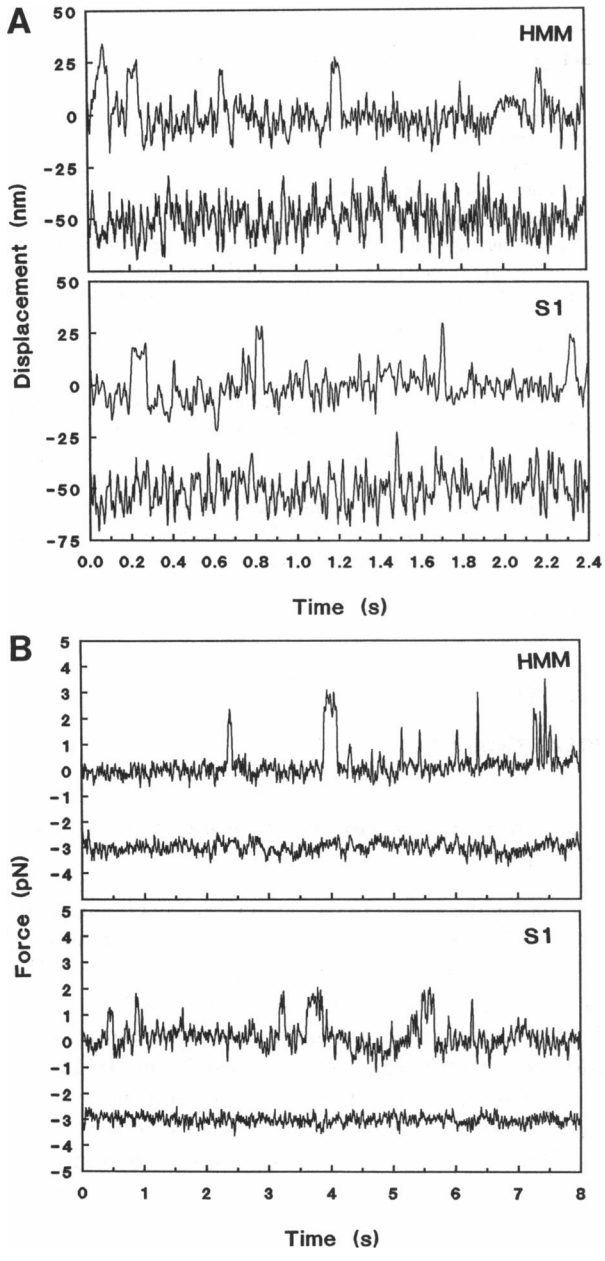

FIGURE 2 Single mechanical interactions between rabbit actin and rabbit HMM or S1 measured at 10 μM ATP (all data were filtered using a 9-point running average). The major noise contribution in all of these records is thermal motion of the bead. The x axis stiffness in these measurements is 0.04 pN/nm, and the y axis stiffness is 0.02 pN/nm. (B) Force records. Traces of force against time. The upper traces are the AOD output parallel to the thin filament (x), and the lower traces that perpendicular (y) (data sample rate 500 Hz). (A) Displacements. Traces of displacement against time. The upper traces are x data, and the lower traces are y data (data sample rate 2 kHz). Because the traps are aligned in the x axis and the actin filament is pulled taut, Brownian noise is less in the x than in the y axis. During attachments, cross-bridge stiffness adds to the overall stiffness, which constrains the beads' thermal motion, so noise is reduced.

from rabbit, *Drosophila* wild type (WT) and *Drosophila* Act88F^{E93K}. The force pulses were significantly smaller in the *Drosophila* actin interactions (Molloy et al., 1995). However, work stroke sizes were the same.

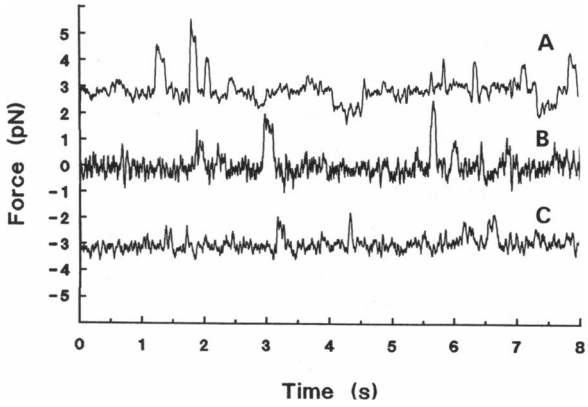

FIGURE 3 Force records of rabbit HMM with: (A) rabbit actin (note the negative forces seen in this record; (B) *Drosophila* WT actin; (C) *Drosophila* ACT88F^{E93K} actin.

Lifetime of attachment: probability of detachment

Fig. 4 shows data of force records for rabbit HMM with rabbit and *Drosophila* WT actin recorded on slow timebases. Also shown are the distribution of the times of attachment, in which attachment is defined as a transition in the force records greater than a threshold (indicated by the horizontal

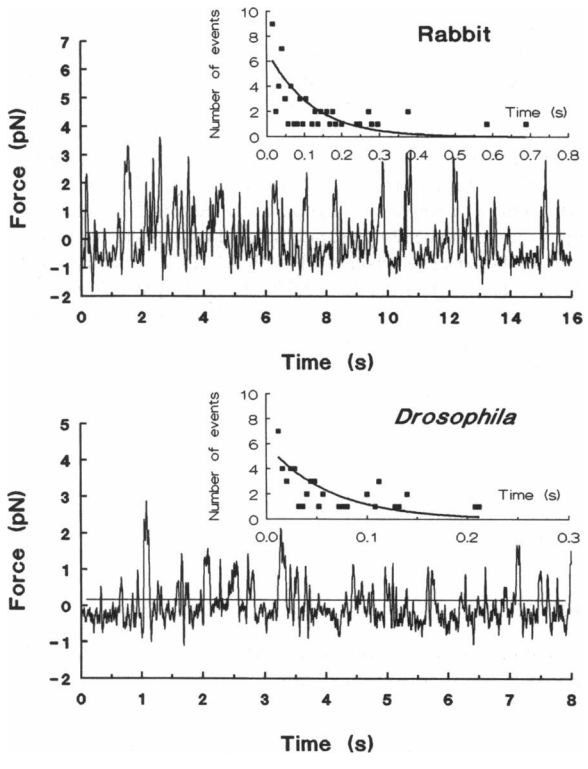

FIGURE 4 Lifetime of attached states. Records of force against time obtained for rabbit HMM interacting with Rabbit and *Drosophila* actins. There is probably more than one head interacting with the actin filaments in these traces. The horizontal lines indicate the threshold used to determine whether a myosin HMM was attached for the analysis of the distribution of attached lifetimes (histograms, *inset*). The exponential fit for the data obtained using rabbit actin has a rate constant of 8.7 s^{-1}. The exponential fit for that using *Drosophila* actin has a rate constant of 16.0 s^{-1}.

lines), lasting for at least 10 ms. The best-fit exponential curves to this data are also indicated; the rate constants from these fits gives the value of the rate constant for detachment. This preliminary result implies that the attached lifetime of rabbit HMM with *Drosophila* WT actin is shorter than with rabbit actin.

Distortion-dependent responses

What is the stiffness of a single cross-bridge? We have measured the stiffness of the interaction between a single S1 and an actin filament by applying a sinusoidal length change of 20-nm peak-to-peak amplitude at a frequency of 2.5 Hz to a thin filament and after the tension changes induced during the attached phase of the cross-bridge. Fig. 5 *A* shows the results from one such experiment. In Fig. 5 *B* length is plotted against tension for the two attached phases indicated in Fig. 5 *A*. The displacement between the traces is due to a greater tension being exerted by the cross-bridge during attachment 1 than attachment 2. The stiffnesses of the two plots are about the same, 0.13 pN nm^{-1}. This estimate is greater than that calculated from the mean force and displacements (1.8 pN/15 nm = 0.12 pN nm^{-1}) but less than measurements made using muscle fibers (Huxley and Simmons, 1971).

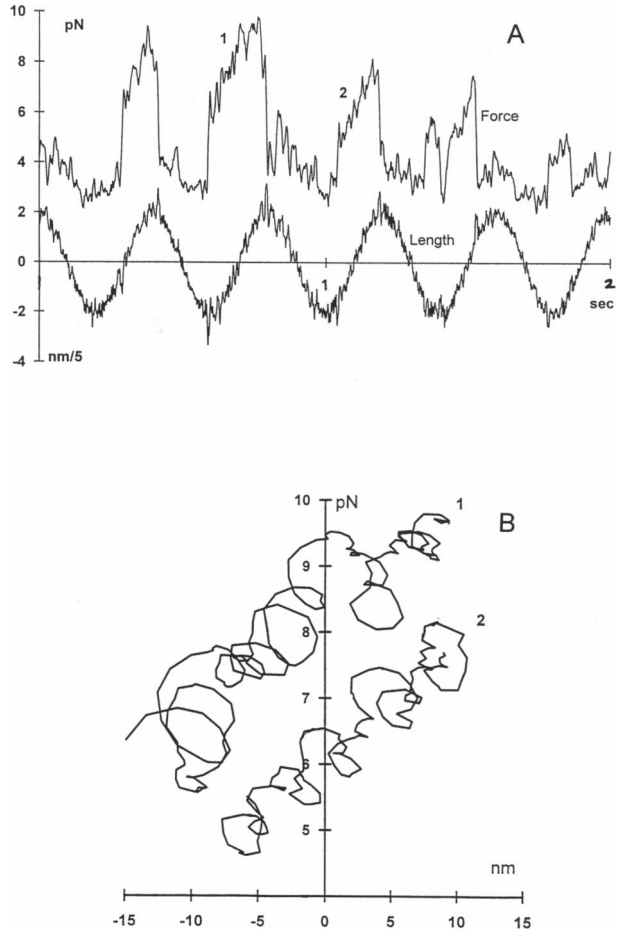

FIGURE 5 Stiffness of S1. (*A*) The lower trace shows the length change applied to the actin filament as a function of time, and the upper trace shows the corresponding trace of force. (*B*) Length against tension for the two attachments labeled 1 and 2 in *A*.

Distortion dependence of cross-bridge detachment

Fig. 6 is a longer time recording including the same trace as Fig. 5. It is apparent from this trace that the lifetime of the attached state during the stretch phase of the cycle is far greater than that during the release phase (*arrowed*). This is a highly qualitative demonstration of a strong distortion dependence of attachment, and the direction of the effect, that pulling on a cross-bridge tends to keep it attached, as is expected from the Fenn effect.

Distortion dependence of attachment (geometric constraints imposed by stereospecific binding)

Fig. 7 *A* shows the results of a preliminary experiment in which a larger periodic length change was applied to the actin filament to move it slowly past a single myosin S1, and the force of interaction between the S1 and the myosin recorded with the trap in force mode (baseline force arising from the stiffness of the other trap has been subtracted away). The length change was triangular, i.e., backward and forward at a constant velocity as indicated in the figure, at a frequency of 0.25 Hz and peak-to-peak amplitude of 100 nm. The velocity of movement was thus 50 nm s^{-1}, which is much less than the free-sliding velocity of actin filaments measured in these assays (0.5–2 μm s^{-1}). Cross-bridge interactions are seen as vertical displacements of the force trace.

To show the positions at which attachment occurred, the data of Fig. 7 *A* are replotted as a trace of force against position in Fig. 7 *B*. It can be seen that attachments occur preferentially at discrete positions (*arrowed*). The separation of the arrows is about 40 nm, which is very close to the period

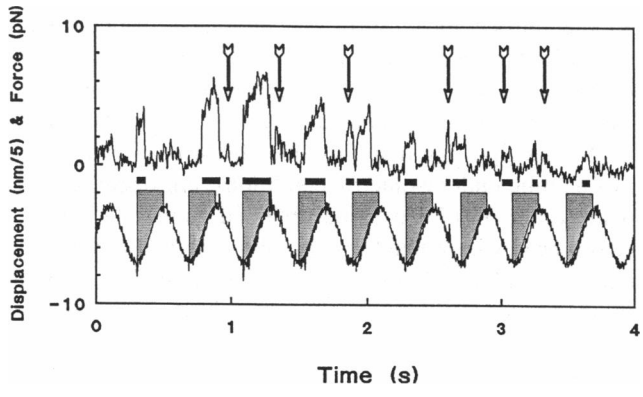

FIGURE 6 Distortion dependence of detachment. A longer record of Fig. 5. The period when the length is increasing is shown shaded. Attachments during the release phase are arrowed. Their duration is much less than that obtained during the stretch phase of the cycle.

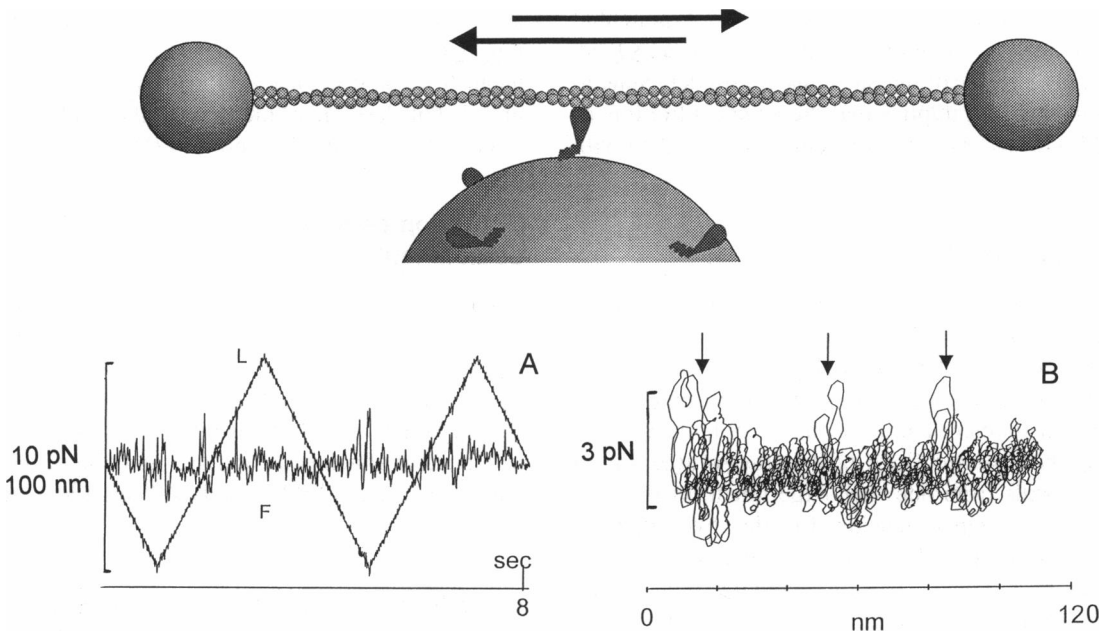

FIGURE 7 Preferred attachment positions on actin filament. Force records obtained during the application of a 100-nm periodic length change applied to a thin filament (shown schematically above). (*A*) Traces of applied length (*L*) and force (*F*) against time. (*B*) Force (*F*) plotted against position (*L*) from *A*.

of the twist of the thin filament of 38.5 nm. Azimuthal attachment selection has been seen in rigor myofibrils (Reedy, 1968), and physiological significance in active muscle has been proposed (Wray, 1979).

DISCUSSION

The results that we have shown are a series of preliminary experiments that demonstrate the capabilities of the optical tweezers apparatus for measuring mechanical and kinetic properties of single-molecule interactions. Finer et al. (1994) showed single molecule interactions between rabbit HMM and rabbit actin filaments. Our findings are in broad agreement with theirs, although we measure slightly longer working strokes and lower mean forces. Lower forces could arise from larger compliance between our myosin subfragments and the nitrocellulose surface, and longer displacements could arise because both their and our data events were analyzed by eye; this may have introduced observer bias.

We show that S1 has very similar forces and displacements to HMM, suggesting that only one HMM head is acting at any one time under isometric conditions. For displacements on the order of 15 nm to be obtained from rotation of a myosin head whose length is also about 15 nm, a change in angle of 1 radian (60°) is required. For an angle change of this magnitude, it is much more straightforward mechanically for this to occur from an angle of less than 90° to one of greater than 90° (see Irving et al., this volume).

The similarity of the results with HMM and S1 suggests that only one head of myosin is required for force production and that myosin S2 is not required per se for force production. The implication is that cross-bridge compliance resides mainly in acto-S1 and not in S2. However, the nature of attachment between myosin subfragments and the nitrocellulose substrate is not clear, and it is possible that myosin-substrate attachment compliance is required for force production.

Frequently, we observe negative force and displacement events in our data (see, for example, Fig. 3 *A*). We believe these events are real and that they could occur in two possible ways. Either a) cross-bridges attach with negative distortion (i.e., pushing rather than pulling) because of thermal motion of the actin filament and myosin head, or b) at low ATP concentrations cross-bridges sometimes undergo futile work strokes, before attachment to actin, and the negative steps are work stroke reversals after attachment. These possibilities can be tested by performing experiments at different ligand concentrations.

Rabbit versus *Drosophila* wild-type and mutant, E93K, actins

Rabbit HMM interacting with *Drosophila* wild-type and E93K mutant actins produced the same displacement as with rabbit actin, but with lower force in wild-type and lower still in E93K. In this case, the work stroke is independent of actin structure, but cross-bridge force or cross-bridge stiffness is greatly affected by the molecular structure of the acto-myosin binding surface. This implies that the acto-myosin binding site is a major source of cross-bridge compliance.

Shorter attached lifetimes found with *Drosophila* wild-type compared with rabbit actin can be explained by lower cross-bridge stiffness (consistent with the lower forces), which would reduce the free-energy barrier for biochemical transitions and, therefore, increase the detachment rate constant.

In other experiments (in collaboration with Azam Razzaq and Roisean Ferguson), we found that the free-sliding velocity of wild-type *Drosophila* actin was slower than rabbit, whereas E93K was slower still and moved only at very low ionic strength. Together with our findings here, this implies that filament sliding velocities can be controlled not by the size of the working stroke but by the amount of force produced by myosin.

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DISCUSSION

Session Chairperson: Kenneth A. Johnson
Scribe: Seth Hopkins

DAVID WARSHAW: I think you have an elegant approach here of getting the stiffness of a single cross-bridge, and the unique aspect is that you are also doing it under low ATP conditions and so therefore you really have rigor bridges because the ADP would have been off and its waiting for an ATP to come along. So you can get stiffness of a rigor bridge, and I would suggest an important experiment. That would be to try to do this under higher ATP conditions to see if you get the same stiffness to finally answer the question: does a rigor bridge stiffness equal that of an active cross-bridge?

JUSTIN MOLLOY: Right, that would be a nice experiment to do.

AMIT MEHTA: Justin, in the sliding filament in vitro assay, S1 supports movement at a greatly reduced speed with respect to HMM by more than a factor of three. I don't recall the numbers, but that doesn't seem like it can be accounted for by the subtle differences in step and almost same isometric force. Effects such as attachment artifact you would think would affect both assays equally. So I wondered if you had any ideas as to why that would be the case.

MOLLOY: I guess that the attachment artifact is probably the answer to that. In this assay, you are selecting a single head and you are selecting on the basis that it is a working head. In the free-running assay, you might have heads stuck all over the place, some of them may be interferring and slowing the filament down. So with the S1, maybe it just doesn't stick down in the right orientation as nicely as the HMM or something like that.

MEHTA: But wouldn't you expect to get alot more small events if that were the case?

MOLLOY: I guess that's right, but when you are hunting around over a bead, basically you have to do a little hunt for events because there is very low density. You are always looking for events and not nonevents. That would be an experiment worth doing: literally probing for a lack of events and scoring them.

HIDETAKE MIYATA: In your measurement of the S1 stiffness, the amplitude of the response curve becomes smaller—why is that? Another question is how do you decide the cutoff level of the duration time?

MOLLOY: The parameters I chose for the forcing function were chosen based on my knowing the half-time of the on time, so I put the sine wave at 2.5 Hz and that seemed to work out quite nicely, and I set the amplitude at 20 nm to give a reasonable distortion while it was bound. But you are quite right in that record you do see a slight tailing off in the effect and maybe that's just drift, that the actin filament is drifting away from the S1. Because if you have 10 nm worth of drift over 8 s, you would see something just like that.