Direct measurement of stiffness of single actin filaments with and without tropomyosin by in vitro nanomanipulation

H. KOHIMA*, A. ISHIIMA*, and T. YANAGIDA*††

*Bio-Motron Project, Exploratory Research for Advanced Technology, Research and Development Corporation of Japan (JRDC), Senba-Higashi 2-4-14, Mino, Osaka; and †Department of Biophysical Engineering, Osaka University, Toyonaka, Osaka, Japan

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ABSTRACT In order to explain the molecular mechanism of muscle contraction, it is crucial to know the distribution of the sarcomere compliance of active muscle. Here, we directly measure the stiffness of single actin filaments with and without tropomyosin, using a recently developed technique for nanomanipulation of single actin filaments with microneedles. The results show that the stiffness for 1-μm-long actin filaments with and without tropomyosin is 65.3 ± 6.3 and 43.7 ± 4.6 pN/μm, respectively. When the distribution of crossbridge forces acting along the actin filament is taken into account, the elongation of a 1-μm-long thin filament during development of isometric contraction is calculated to be =0.23%. The time constant of force in response to a sudden length change is <0.2 ms, indicating that the viscoelasticity is negligible in the millisecond time range. These results suggest that =50% of the sarcomere compliance of active muscle is due to extensibility of the thin filaments.

In striated muscles, contraction is due to cyclic interactions of myosin crossbridges with thin filaments in the sarcomere (1, 2). The mechanical properties of the interaction have often been studied by analyzing tension transients of contracting muscle fibers. In 1971, Huxley and Simmons (3) deduced an ingenious mechanical model to fit the kinetics of these transients. In this model, the force produced by the crossbridges is transmitted to thick and thin filaments by a series elastic element. The model assumed for simplification that the mechanical compliance (reciprocal of the stiffness) in the sarcomere is mostly localized in the crossbridges and that the thin and thick filaments are rigid to transmit the force produced by the crossbridges to the next sarcomere along the muscle fiber axis. This assumption has allowed us to lump the mechanical behavior of the crossbridges distributed along the thin filaments. Indeed, many subsequent experiments have been interpreted on the basis of this simplifying assumption.

The assumption that compliance resides primarily within the crossbridges has been tested by many mechanical and structural studies. Ford et al. (4) measured the instantaneous stiffness of isometrically contracting muscle fibers at various sarcomere lengths and concluded that ≥80% of the sarcomere compliance is due to the crossbridges. A similar result was obtained from the dependence of the rigor stiffness on the sarcomere length (6). Huxley and coworkers (7, 8) showed that the strong 5.9-nm x-ray diffraction peak from actin filaments did not change spacing significantly during contraction. These results suggested that the thin filaments are rigid, but the experiments are not conclusive (see Discussion). On the other hand, measurements of the elastic modulus for bending of actin filaments have suggested that the actin filament is quite flexible and, hence, might contribute significantly to muscle compliance (see ref. 9 for review).

Recently, we have developed a nanomanipulation technique for single actin filaments that involves attaching them to microneedles and observing the bending of the needle under a microscope. This approach enables us to exert and detect forces or displacements on an actin filament with subpiconewton or subnanometer resolution at submillisecond time resolution (10). Using this technique, we have measured the stiffness of actin filaments with and without tropomyosin. The results show that the actin–tropomyosin complex is much more compliant than was assumed in previous crossbridge models (3). Preliminary results have been reported (11).

MATERIALS AND METHODS

Sample Preparation. All proteins were obtained from back skeletal muscle of rabbits. G-actin was extracted from acetone powder and purified (12). Tropomyosin (13) at 0.5 mg/ml was bound to actin at 1 mg/ml in 150 mM KCl/20 mM Hepes, pH 7.8. Actin or actin–tropomyosin filaments (2.5 μm actin monomers) were labeled with phallolidin-tetramethylrhodamine (PHDTMR) by overnight incubation at 4°C in 5 μM PHDTMR/100 mM KCl/20 mM Hepes, pH 7.8 (14). Just prior to use, the filaments were diluted to 8.3 nM final actin monomer concentration. Myosin was extracted from muscle (15) and purified (16). For storage, myosin was rapidly frozen with liquid nitrogen and kept at −80°C. All experiments were performed in a medium containing 25 mM KCl, 3 mM MgCl2, 20 mM Hepes (pH 7.8), 0.5% 2-mercaptoethanol, and oxygen-removal reagents (glucose, 2.3 mg/ml; catalase, 0.018 mg/ml; glucose oxidase, 0.1 mg/ml) at 25–27°C.

Preparation of the Microneedles. Glass microneedles, 0.3 μm in diameter, were made from 1-mm-diameter glass rods with a glass-electrode puller (PD-5; Norishige, Tokyo). A microneedle was attached to a rigid glass rod (tip diameter, =100 μm) with epoxy resin and cut by a heated 5-μm-diameter platinum wire under a binocular microscope. The lengths of the microneedles were 70–100 μm. To increase contrast, a nickel particle, about 2 μm in diameter, was attached to the tip (10). The stiffness of the microneedle (KN = 0.5–5.4 pN/μm) was determined by cross calibration against a standard needle of known stiffness essentially as described (17). The stiffness of the standard needle was determined by measuring vertical bending due to the weight of 1.2- to 2.4-μg, 25-μm-diameter, 300–600-μm-long steel wires. (The stiffness of the needles was also determined from mean squared thermal vibration ⟨x2⟩ from the relation KN(x2) = kBT, where kB is Boltzmann’s constant and T is absolute temperature (10). The values of KN measured by thermal vibrations were similar (87–100%) to those determined from the cross calibration.) The relaxation time of the needle tip upon a sudden length change, τs (= Ks/ξN, where ξN is the needle’s frictional coefficient in solution) was

Abbreviation: PHDTMR, phallolidin-tetramethylrhodamine.

†To whom reprint requests should be sent at the † address.

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estimated from the corner frequency of the power density spectra of the thermal vibration of the needles, \( f_c \), as \( \tau_N = (2\pi f_c)^{-1} \). \( \tau_N \) was \( \approx 0.3 \) ms at \( K_N = 0.5 \) pN/nm and \( \approx 0.03 \) ms at \( K_N = 5 \) pN/nm (details will be published elsewhere). The microneedle was coated with myosin monomers to increase its affinity for an actin filament by a rigor complex (17).

Apparatus. Manipulation of single actin filaments by microneedles and measurement of nanometer-range displacements with submillisecond time resolution were performed with essentially the same apparatus recently developed in our lab (10) (Fig. 1A) the schematic diagram of the apparatus. The setup was built on an inverted fluorescence microscope and the needles were manipulated by fine hydraulic micromanipulators and piezo actuators. The image of a nickel particle attached to the tip of a needle (about 500 \( \mu \)m in diameter after magnification) was projected onto a differential pair of photodiodes (each with dimensions of 1030 \( \mu \)m \( \times \) 1010 \( \mu \)m) that were separated by a gap of 30 \( \mu \)m. The signal output was very sensitive to motions of the needle tip, and displacements of \( <1 \) nm could be detected. The response time of this system was 0.03 ms. The differential photodiode signal was recorded on tape by a digital data recorder (RD101T; TEAC, Tokyo) and analyzed by a signal processor (R9211A; Advantest, Tokyo) and a computer (PC-9801; NEC, Tokyo).

RESULTS

Measurements of Elasticity of Actin Filaments. The ends of a single actin filament, which had been labeled with PH- DTMR, were caught by two types of glass needles. One was very fine (\( \approx 0.3 \) \( \mu \)m in diameter and 70–100 \( \mu \)m in length) for measuring the displacement and force, and the other one was relatively thick (\( \approx 2 \) \( \mu \)m in diameter) for rigidly holding the actin filament. Sinusoidal displacements with 10-nm peak-to-peak amplitude and frequency of 20 Hz were applied to the base of the fine needle by a piezo actuator (Fig. 1A). Fig. 2A shows the sinusoidal voltage change applied to the piezo actuator, and Fig. 2 B and C show the recordings of the movements of the tip of a fine needle when the needle is free (\( x_0 \)) and attached to an actin filament (\( x \)), respectively. The amplitudes of the needle movements were determined from the peak heights of their power spectra (Fig. 2 D and E). Let \( K_e \) be the composite stiffness of the actin filament and its attachment to the needle. Then the peak-to-peak force is given by \( F_p = xK_e \). The force is also given by the product of the needle deflection and the needle stiffness, \( F_p = (x_0 - x)K_N \). Setting the two expressions for force equal, we obtain \( K_e = (x_0/x - 1)K_N \) (see Fig. 1B and Appendix).

The actin filament was attached to the needles through myosin bound to the surface. Therefore, the bonds between the actin filament and the needles were not completely rigid. To separate the stiffness of actin filaments from that of these bonds, we measured the composite stiffness for actin filaments over a length range of 3–30 \( \mu \)m. The composite stiffness, \( K_c \), is given as \( 1/K_c = (L/K_a) + (1/K_b) \), where \( K_a \) is the stiffness for a 1-\( \mu \)m-long actin filament, \( K_b \) is the stiffness of the bonds, and \( L \) is the length of the actin filament (see Fig. 1B). Thus, the values of \( K_a \) and \( K_b \) can be obtained from the slope and intercept of a plot of \( 1/K_c \) vs. \( L \), respectively.

Fig. 3 shows plots of \( 1/K_c \) vs. \( L \) in the presence and absence of tropomyosin. As expected, the relationship of \( 1/K_c \) vs. \( L \) is linear. From the intercept of the lines, \( K_b \) in the absence and presence of tropomyosin was 5.7 \( \pm \) 1.2 and 5.3 \( \pm \) 0.6 pN/nm (mean \( \pm \) SEM), respectively. From the slopes, the stiffness of actin filaments with and without tropomyosin was 65.3 \( \pm \) 6.3 (mean \( \pm \) SEM, \( n = 116 \)) and 43.7 \( \pm \) 4.6 pN/nm (\( n = 74 \)), respectively. The standard deviations of \( 1/K_c \) were rather large (Fig. 3), probably because of variation in the stiffness of the bonds, \( K_b \). Although the actin filaments were attached perpendicular to the fine needle in order to make the length of the actin filaments interacting with myosin on the needles (\( \approx 0.3 \) \( \mu \)m) as constant as possible, there is probably some variability in this value from experiment to experiment. Since the attachment of actin to the thick needle was \( >2 \) \( \mu \)m long, the compliance associated with this attachment should be negligible (Fig. 1A).

Viscoelastic and Force-Dependent Natures. To examine the viscoelastic nature of the actin filament, we compared the phase lag between the sinusoidal motion of the tip of the fine
a signal processor. When sinusoidal length changes with frequencies of 10 to 500 Hz were applied to the base of the fine needle, the phase difference, \( \Delta \phi = \phi - \phi_N \), was <0.1 radian (Fig. 4). (The Appendix describes the use of these data to estimate the viscoelastic characteristics of the actin filament.) The stiffness \((K_N)\) and relaxation time \((\tau_N)\) of the needle used were \( \approx 5 \) pN/nm and \( \approx 0.03 \) ms, respectively. If \( f = 500 \) Hz, \( \phi_N = 2\pi f \tau_N = 0.09 \) radian. Thus, \( \phi = \Delta \phi + \phi_N < 0.1 + 0.09 = 0.19 \) radian (see Appendix). The composite stiffness, \( K_c \), was \( \approx 1.6 \) pN/nm when the filament length, \( L \), was 20 \( \mu \)m (Fig. 3). By inserting these values into Eq. 5 in the Appendix, \( \tau_c \) is found to be <0.1 ms. By inserting this value and the values of \( K_0 \) (44 pN/nm) and \( K_b \) (5.7 pN/nm) (Fig. 3) into Eq. 6, \( \tau_c \) is <0.2 ms at \( L = 20 \) \( \mu \)m. Thus, the time constant of force response to a rapid length change applied to the actin filament is <0.2 ms. If the length of the filament was similar to that in skeletal muscle (i.e., \( L = 1 \) \( \mu \)m), then \( \tau_c (= \xi_4 L/K_0) \) would be <0.02 ms.

The composite stiffness was measured at forces of 35–170 pN, which corresponded to 15–70% of the force exerted on the thin filament in isometrically contracting muscle (230 pN; ref. 4) (Fig. 5A). The forces were applied to the filament by moving the thick needle as in Fig. 2. The stiffness was approximately constant (Fig. 5B). In this experiment, the stiffness of the actin filament was not separated from that of its attachment to the needles. Since it was unlikely that changes of the stiffness of the filament and its attachment canceled each other, however, the results indicate that the stiffness of actin filament is almost constant over the entire force range examined.

**DISCUSSION**

Comparison with Elasticity of Other Proteins. 

For comparison with the elasticity of other proteins, a rough estimate of Young’s modulus of an actin filament is given. Although the actin filament is made up of actin monomers polymerized into a double-helical form, we assume that the actin filament is a homogeneous rod. Young’s modulus for a homogeneous rod, \( E \), is given as \( E = K(L/S) \), where \( K \) is the stiffness for actin filament, \( L \) is the length of the filament, and \( S \) is the average cross-sectional area of the filament. The value of \( K \) at \( L = 1 \) \( \mu \)m was found to be \( 43.7 \pm 4.6 \) pN/nm. The average cross-sectional area, \( S \), is \( \approx 25 \) nm\(^2\) from the average contour of the filament cross section based on the atomic model of the filament (18, 19), if the area in the contour is filled homogeneously. Thus, \( E \) is calculated to be \( \approx 1.8 \times 10^9 \) N/m\(^2\). This

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**Fig. 2.** Records of the needle displacements and the power spectra. (A) A sinusoidal voltage change applied to the piezo actuator. \( f = 20 \) Hz. \( (B \) and \( C) \) Displacements of the tip of the fine needle when the needle was free (\( \phi_N = 10 \) nm) and attached to the filament (\( \phi \)), respectively. The tip was pulled taut by moving the thin filament with a piezo actuator before applying the sinusoidal length change. The force exerted on the filament by pulling it was 190 pN. Length of the actin filament was 23 \( \mu \)m. The stiffness of the needle used in this measurement was 1.7 pN/nm. Traces show averages of 32 runs. \( (D \) and \( E) \) Power spectra of traces shown in \( B \) and \( C \). Power spectra were integrated for 8 s.

**Fig. 3.** Relationship between the reciprocal values of composite stiffness (1/\( K_c \)) and the filament lengths (\( L \)). \( \bullet \) Actin filaments. \( \bigcirc \) actin–tropomyosin complexes. Bars indicate standard deviation (\( n = 4-26 \)). Means were obtained every 3 \( \mu \)m from the shortest filaments. Solid lines were obtained by least-squares regression for all data (\( n = 74 \) for actin filaments; \( n = 116 \) for actin–tropomyosin complexes). Temperature was 25–27°C.

**Fig. 4.** The difference (\( \Delta \phi \)) between the phase lags of sinusoidal motions of the needle tip attached to the actin filament (20 \( \mu \)m) when free. Sinusoidal length changes with a 100-nm peak-to-peak amplitude and various frequencies were applied at the base of the needle. Bars indicate standard deviation (\( n = 5-8 \)). Lengths of the actin filaments were 3.8–21 \( \mu \)m.
value is similar to that deduced from the thermal bending motions in solution of actin filaments labeled with phalloidin-rhodamine (14, 20). Young’s modulus for actin filaments is also close to that of other proteins such as silk (<7 × 10^6 N/m^2; ref. 21) and collagen (<5 × 10^6 N/m^2; ref. 22).

When tropomyosin was added to actin filaments, the stiffness increased to 65.3 ± 6.3 pN/nm. Tropomyosin forms a double-stranded coiled coil of nearly 100% α-helix, and its length is 40 nm (23). Tropomyosin molecules bind to each groove of the actin filament helix and join end-to-end. Therefore, the stiffness of tropomyosin (two molecules) for a 1-μm-long filament is obtained simply as the difference between the stiffness of an actin–tropomyosin complex and an actin filament, 21.6 pN/nm, if we assume that the binding of tropomyosin does not affect the elastic properties of the actin itself. Since the radius of an α-helix is 0.4–0.5 nm (24), Young’s modulus of tropomyosin in the longitudinal direction is calculated as E = 1 × 10^{10} N/m^2, several times larger than that of the actin filament, as expected from the high α-helical content. This value for tropomyosin is several times larger than that of the α-helix region in wool (1.2 × 10^9 N/m^2; ref. 25) and myosin subfragment S-2, deduced from bending motions (1.3 × 10^9 N/m^2; ref. 26), but similar to that of an α-helix estimated by theoretical analysis of its dynamic structure (7 × 10^9 N/m^2, ref. 24).

Implications for Sarcomere Compliance in Muscle. The elongation of thin filaments in isometrically contracting muscle fibers can be estimated from the stiffness of actin–tropomyosin filaments. In the steady state, in which active crossbridges are cycling between attachment and detachment, the force (F) exerted on a thin filament is expected to be linearly proportional to the length (l) of the overlap region between the thin and thick filaments. Then, the steady-state elongation

\[ \Delta Y = (l_0 - l) \frac{F}{K_a} + (\frac{l}{2}) \times (F/K_a) \]

where \(K_a\) is the stiffness of the actin–tropomyosin complex and \(l_0\) is the length of the whole thin filament (27). F is 230 pN for frog skeletal muscle (4), and \(l_0\) and \(l\) have been reported to be 1.0 μm and 0.7 μm, respectively (28, 29). With these values in the above equation, the steady-state elongation, \(\Delta Y\), is 2.3 nm. The elongation of the thin filaments in the overlap region would not be dissipated immediately upon sudden shortening of the active muscle, since thin filaments are bound to myosin heads. The shortening of the thin filament in the overlap region is determined not only by the stiffness of thin filaments but also by the viscoelasticity of the crossbridges (4, 27). The instantaneous shortening (\(\Delta \gamma\)) required to reduce force to near zero in an isometrically contracting muscle was found to be \(\approx 3.9\) nm per half-sarcomere (30). From equation A 10 of Ford et al. (4), the contribution of the thin filaments to \(F_\gamma\) (3.9 nm) is estimated to be 1.9 nm. Thus, 49% of the instantaneous sarcomere compliance in active muscle is due to the thin filaments, while remainder is due to the crossbridges and thick filaments. As pointed out by Ford et al. (4), if the actin filament were viscoelastic, the effective stiffness would be higher under conditions of rapid length changes. However, this is not the case, because the viscoelasticity of actin filaments is negligible in the submillisecond range (Fig. 4).

**Relations to Other Studies.** Mechanical studies on muscle fibers have suggested that the contribution of both thin and thick filaments to the sarcomere compliance is <20% (3, 4, 6). If this is correct, then either native thin filaments (plus other associated proteins such as nebulin) are much stiffer than the actin–tropomyosin complex formed from purified proteins or phallolidin binding makes actin filaments more elastic. Recently, the stiffness for 1-μm-long nebulin filaments in myofibrils has been estimated to be \(1.03 \times 10^2\) pN/nm (31). The nebulin, therefore, would contribute little to the sarcomere stiffness. Since phallolidin stabilizes the filament structure of actin (32), it is also unlikely that it makes the actin filament more elastic. Thus, we consider these possibilities to be unlikely. The extensibility of thin filaments in muscle was determined by measuring the dependence of the active fiber stiffness on the sarcomere length. However, this experiment is difficult because it requires accurate detection of small changes (<0.01% nm) in sarcomere lengths during the time course of length changes applied to the fiber to measure stiffness. Julian and Morgan (33) and Bagni et al. (34) suggested somewhat larger compliance of thin filaments (30% and 19% for total sarcomere compliance, respectively) than Ford et al. (4). Recently, Higuchi et al. (37), using skinned muscle fibers with homogeneous sarcomere lengths, measured the rigor stiffness and monitored sarcomere length very accurately by a white-light diffraction method (35). The results showed that the contribution of thin filaments to muscle fiber compliance in rigor is ~50%, a value which is consistent with our in vitro data.

The extensibility of thin filaments in muscle has also been recently reexamined by x-ray diffraction. In the earlier studies (7, 8), the extensibility of thin filaments was analyzed by measuring spacing of the strong 5.9-nm reflection. Those studies failed to detect extensibility of actin filaments, but the 5.9-nm spacing may not be a good indicator of the extensibility and the spatial resolution of those studies also may not
have been sufficient. The use of a very intense x-ray beam from synchrotron orbital radiation has now allowed detection with high spatial and temporal resolution of the 2.7-nm diffraction peak, which is directly related to the extensibility (7), during isometric contraction. Two groups have independendly obtained evidence that the extensibility of actin filaments is 0.3-0.4% (3-4 nm in elongation) during isometric contraction (5, 36).

The results suggest that 50% of the sarcomere compliance of active muscle is due to thin filaments. In other work, the compliance of thick filaments has also been suggested to be not so small as to be neglected (5, 36). Thus, models based on the assumption that the sarcomere compliance resides mostly in the crossbridges may need to be reconsidered.

**APPENDIX**

When a sinusoidal length change with a low frequency is applied to the base of a needle, the movement of the tip attached to the filament is given as

$$x = x_0 K_0/(K_N + K_C),$$  

[1]

where $x_0$ and $x$ are the displacements of the tip and the base of the needle, respectively; $K_0$ is stiffness of the needle, and $K_C$ is the composite stiffness of the filament and the filament-needle bonds:

$$K_C = [(x_0/x) - 1]K_N.$$  

[2]

In the experiments, $x_0$ was obtained from the displacement of the tip when the needle was free, because the attenuation of the displacement of the tip due to viscous drag in solution was negligible at low frequency. For instance, at $f = 20$ Hz, the attenuation factor $[1 + (2\pi f r_N)^2]^{-1/2} = 0.998$ and 0.9999998 for $K_N = 0.5$ and 5 pN/nm, respectively.

When the frequency $f$ is high, the viscoelasticities of the needle, the filament, and the bond cannot be neglected. In this case, expressing displacements and viscoelasticity as phasors, the displacement is given as

$$x = x_0 K_N/(K_N + j2\pi f \xi_N + j2\pi f \xi_C),$$  

[3]

where $x_0 e^{j2\pi f t}$, $\xi_N$ is the frictional coefficient of the needle, and $\xi_C$ is the viscoelastic coefficient of the composite filament and bond. In the experiments (Fig. 4), to cancel the phase lag due to the electric circuit, $\phi_0$, which was 0.09 radian at $f = 500$ Hz, we obtained the difference between the phase lags of the signal output of the sinusoidal motions of the tip of the needle attached to the filament ($\phi + \phi_0$) and the free needle ($\phi_N + \phi_0$), $\Delta\phi = (\phi + \phi_0) - (\phi_N + \phi_0) = \phi - \phi_N$. And $\phi$ is obtained as $\phi = \Delta\phi + \phi_N$. The phase lag, $\phi$, is given as

$$\phi = \tan^{-1} 2\pi f (\xi_N + \xi_C)/(K_N + K_C).$$  

[4]

When we rearrange this equation, the time constant of composite stiffness, $\tau_C$ (i.e., $\xi_C/K_C$), is obtained as

$$\tau_C = (K_N/K_C)[(1 + (K_C/K_N)) (2\pi f)^{-1} (\tan(\phi) - \tau_N)],$$  

[5]

where $\tau_N = (\xi_N/K_N)$ is the time constant of the free needle. Similarly, the time constant of the actin filament, $\tau_a$, is obtained as

$$\tau_a < [1 + (K_a/K_B L)]\tau_c,$$  

[6]

where $K_B$ and $\xi_B$ are the stiffness and viscoelastic coefficient of the bond, respectively.

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