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On the stiffness of the natural actin filament decorated with alexa fluor tropomyosin

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Abstract

Natural, phalloidin-free, actin filaments were decorated with tropomyosin made fluorescent by reaction with alexa fluor (R) 488 C_5 maleimide. The elastic modulus by stretching of these filaments was then determined and found to span between 38.2 MPa and 61.48 MPa. We tried also to determine the yield strength of the same filaments in the laser light trap operated at 920 mW, the maximum power of the apparatus. Only two out of the 10 filaments tested were broken under these conditions, yield strength being 50.5 and 55 pN, respectively.

Keywords: Actin; Tropomyosin; Elastic properties

1. Introduction

The mechanical properties of phalloidin F-actin were extensively investigated by the Yanagida group. Tensile strength was measured and found to be 430 pN [1], to be independent of the length of tropomyosin-decorated phalloidin F-actin between 5 and 30 μm [2] but to be strongly dependent on the torsion applied to the filament (230 pN for a torsion of 360°) [1]. Surprisingly, no consideration of the influence of critical concentration on tensile strength was made. In fact, theoretical considerations predict that critical concentration (i.e. the dissociation constant of the elongation reaction) is the main determinant of the free energy of the monomer–monomer interaction [3–5]. We directly investigated this aspect by determining tensile strength of phalloidin rhodamine F-actin as a function of the critical concentration [6]. At the conventional ionic strengths, the phalloidin F-actin critical concentration is so low as to be practically not determinable. Therefore, we worked at low ionic strength, where critical concentration is easily determined. We found tensile strength to change inversely with the critical concentration and to increase directly (5.6–28.6 pN) for the increase of ionic strength from 3 to 19 mM [6]. Extrapolation of these data to 100 and 150 mM ionic strengths leads to calculated yield strengths of 165 and 247 pN, respectively, values close to that found by the Yanagida group for phalloidin F-actin at the physiological ionic strength [1,2].

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The finding that critical concentration is the main determinant of the yield strength of F-actin raised the question of how thin filament bears the forces developed in muscle contraction. The critical concentration of F-actin in 0.1 M KC1 and 2 mM $MgCl_2$ is approximately 330 nM and even with the contribution of tropomyosin [7] and the increase of the macromolecular osmotic pressure [8], it cannot be expected to reach the values of the critical concentration of rhodamine phalloidin F-actin (30 nM) observed in our experiments. In turn, the largest yield strength experienced in our work for rhodamine phalloidin F-actin is $\sim$28 pN, i.e. the contribution of only three to four attached crossbridges [9].

We found that the actin filament, decorated with tetramethylrhodamine (without phalloidin) and at 0.15 M ionic strength displays a yield strength of only 3.5 pN (2 orders of magnitude lower than phalloidin F-actin) [10], With the same filament we showed also that the yield strength increases from 3.5 to 10 pN after decoration with tropomyosin and from 3.5 to 15 pN after decoration with myosin subfragment-1 [11].

In the present work we investigate the elastic properties of natural actin filaments, phalloidin-free and chemical modification free, made detectable by interaction with fluorescent tropomyosin, obtained by coupling with alexa fluor (R) 488 C_5 maleimide.

2. Experimental

G-actin [12] and tropomyosin [13] were from rabbit muscle. Heavy meromyosin was obtained by chymotryptic digestion of myosin [14] and further modified by reaction with *N*-ethylmaleimide [15]. Alexa fluor (R) 488 C_5 maleimide was purchased by Molecular Probes Europe BV, The Netherlands. Stock solutions of Alexa Fluor were prepared in dimethylformamide (Uvasol grade) and kept in the dark at -20 °C.

Protein sedimentation was performed in the Beckman TL100 rotor of the Beckman TL100 centrifuge for 10 min at $360{,}000 \times g$. Total protein and protein in the supernatant solutions were measured either by the method of Bradford [16] or according to Lowry et al. [17].

The fluorescence of alexa fluor tropomyosin and of its complexes with F-actin was measured with a 90° observation angle by using a Jasco FP 550 spectrofluorimeter equipped with a Linseis recorder. The exciting wavelength was 493 nm; the emitting wavelength was 516 nm, excitation and emission bandpasses were 5 nm.

2.1. The labelling of tropomyosin with alexa fluor (R) 488 C_5 maleimide

To a water solution (1 ml) of rabbit muscle tropomyosin, 6 mg/ml, 0.1 ml of 1 M 2-mercaptoethanol was added. After 6 h of incubation at 20 °C, the solution was dialysed for 24 h against three changes of a solution containing 2 mM phosphate buffer, 1 mM ascorbate and 1 mM NaN_3, pH was 7.7.

To the dialysed solution (1.0 ml) 716 mg/ml of guanidinium hydrochloride were added, followed by five 0.01 ml additions of 13.9 mM alexa fluor at 30-min intervals. After 17 h of incubation in the dark at 20 °C, the reaction was stopped by the addition of 2 mM 2-mercaptoethanol (final concentration) and dialysed for 24 h against two changes of a solution containing 5 mM Tris–HCl buffer, 2 mM 2-mercaptoethanol and 1 mM NaN_3, pH 7.5. To the dialysed solution (1.9 ml) 923 mg of ammonium sulfate were added and the solution was centrifuged. The protein pellet was then dissolved with 0.5 ml of a solution containing 5 mM Tris–HCl buffer, 2 mM 2-mercaptoethanol and 1 mM NaN_3, pH 7.5 and extensively dialysed against the same buffer. Tropomyosin in the final solution (1.1 ml) was 4.25 mg/ml as assayed by the Lowry method [17] with native tropomyosin as a standard. The degree of labelling of tropomyosin was found to be 2.9 mol of dye per mol of tropomyosin, by making use of an alexa fluor molar extinction coefficient of $\varepsilon_{493} = 61{,}000$.

2.2. Coating polystyrene microspheres with N-ethylmaleimide-meromyosin

Polystyrene microspheres (diameter 1 μm, Polysciences Inc.) were washed in 0.1 M bicar-

bonate buffer, pH 9.6, resuspended and washed three times in 20 mM phosphate buffer, pH 4.5. The microspheres (1 ml, 2.5% w/v) were then treated with a 1.2-ml solution (2% w/v) of 1-(3-dimethylaminopropyl-3-ethyl carbodiimide hydrochloride in 20 mM phosphate buffer, pH 4.5. After 3–4 h of incubation, with stirring, microspheres were centrifuged, resuspended and washed three times with 0.2 M borate buffer, pH 8.5. Microspheres were then coated with *N*-ethylmaleimide-meromyosin (1 mg/ml) by incubation for 20 min at 20 °C in 0.3 M KC1, 5 mM $MgCl_2$, 25 mM phosphate, pH 7.4.

2.3. The construction of the assay cells

Assay cells were constructed by assembling two glass cover slips in a recessed specimen holder. A cover slip surface was coated with *N*-ethylmaleimide-meromyosin treated microspheres by incubation for 15 min at 4 °C with the assay buffer (15 mM orthophosphate, 3 mM $MgCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol, pH 7.0), treated for 20 min at 4 °C with a 1% solution of bovine serum albumin and rinsed twice with the assay buffer before the introduction in the holder. The cell was then filled with 0.2 ml of a solution containing 20 nM alexa-fluor tropomyosin labelled F-actin plus 0.05% (w/v) *N*-ethylmaleimide-meromyosin treated microspheres in the assay buffer. To minimise photobleaching an oxygen depletion system (4.5 mg/ml of glucose, 230 μg/ml of glucose oxidase and 36 μg/ml of catalase, final concentrations) was added to the solution in all the experiments. Finally, the second cover slip, also treated with bovine serum albumin, was placed over the holder.

2.4. Microscopy and laser light trap

The holder was placed in the stage of an Olympus IX70 microscope equipped with a PLANAPO 100 X, N.A. 1.4 objective and a step motor driven $x-y$ translation stage control (Luis Neuman, Goettingen, Germany). The vertical, z-axis, position was adjusted by changing the focus. The specimen cell was illuminated in bright field by a 100 Watt halogen lamp and in epifluorescence by a 75 Watt Xenon lamp. The images were captured by an Hammamatsu Newicon camera, recorded and stored on videotape. The single beam laser trap was generated by a Ti:Saph laser (Spectraphysics, Grenoble, France) pumped by a 2017S 6 Watt Argon laser (Spectraphysics, Grenoble, France). Experimental temperature (22 °C) was not significantly increased by the irradiation.

2.5. Trap stiffness calibration

The Stokes' force method was used to calibrate the force associated to the displacement of the microsphere from the centre of the trap. The microspheres were of the calibration grade; their diameter was 0.914 ± 0.027 μm. A viscous drag, or Stokes' force was applied to a captured microsphere by moving the specimen holder along the x-axis in both versos [18]. This action promoted the displacement of the microsphere from the centre of the trap. The Stokes' force, F (dyne), on the microsphere was calculated by:

$$F = 6\pi\eta r v$$

where η (Poise) is the viscosity; r (cm) is the radius of the microsphere; and v (cm s^{-1}) is the velocity of the displacement. When the velocity of the stage was increased from 13 to 125 μm s^{-1} the Stokes' force increased from 0.124 to 1.184 pN. The linear relationship between microsphere displacement and Stokes' force was used to estimate the stiffness of the trap. Calibrations and measurements were made at constant vertical height (6500 nm) in the assay chamber. The stiffness of the trap was found to be the same in the $x-z$ plane as well as in the $y-z$ plane and was increasing by 0.000284 pN nm^{-1} for the increase of 1 mW of the power of the trap (Fig. 1). The force on the microsphere at the trap was calculated as the product of the stiffness of the trap and the distance of the microsphere from trap centre. The maximum force applicable by the laser optical was approximately 59 pN and is given by:

$$0.000284 \text{ pN nm}^{-1} \text{ mW}^{-1} \times 920 \text{ mW} \times 225 \text{ nm} = 58.7 \text{ pN}$$

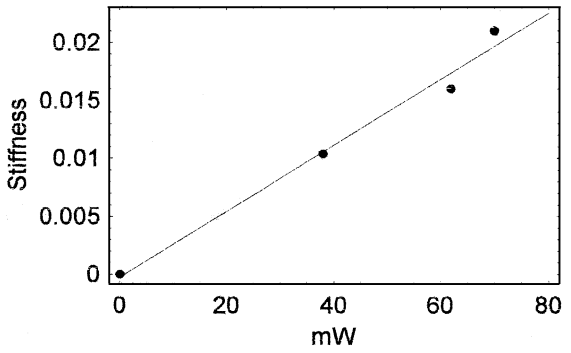

Fig. 1. Relationship between stiffness and power of the trap. Data are fitted by the equation: stiffness (pN nm^{-1}) = 0.000245 + 0.000284 mW.

where 920 mW is the maximum power of the trap and 225 nm is the distance from the centre of the trap beyond which the microsphere escapes the trap.

As an example, the stretching of the alexa fluor-tropomyosin decorated actin filament (n 9) is presented. Power was 920 mW and stiffness 0.26 pN nm^{-1}. The system was first observed at rest (0–b) then the displacement of the stage was started (b–f). The filament broke at point f when applied force was 50.5 pN. Breaking was signalled by the sudden return of the microsphere at the centre of the trap (Fig. 2). How do we know that filaments are breaking far from their ends? As a matter of fact, after breaking, filament tails are bound to the bead at the focus. Of course we do not know whether the relicts are part of the filament (filament breaking) or the whole filament (breaking at the bead fixed at the lower coverslip). However, we exclude this latter possibility because it would imply the unlikely event of the rupture at the connection of the filament with the distal bead only and not also at the connection with the bead at the focus.

2.6. Determination of the length of the filaments

While the force applied to the filaments was estimated from the displacement of the trapped microsphere from the centre of the trap, the length of the stretched filaments was detected directly by recording the position of both microspheres. Accordingly, one end of the actin filament was attached to a *N*-ethylmaleimide-meromyosin treated microsphere, suspended in the assay solution and trapped in the focus of the laser beam, the other end of the filament was attached to a coated bead fixed to the lower coverslip. While the z-axis was focusing fixed, the microscope stage was moved either along the x or the y axis until the filament was broken. The process was followed both with the fluorescent and the bright field control. The stored bright field images of both the beads were analysed by specific softwares.

The length of the filaments was calculated by the following formula:

$$\text{length(nm)} = \text{SQRT}\left[(x_C - x_F)^2 (y_C - y_F)^2 + z_F^2\right]$$

where SQRT indicates the square root, x, y and z indicate the co-ordinates; the subscripts, C and F indicate the microsphere at the lower coverslip and the microsphere at the focus. The co-ordinate z was kept constant at 6500 nm. Due to the noise of recording and video, effective resolution was approximately 15 nm.

Curve fitting was performed by the 'Mathematica' software.

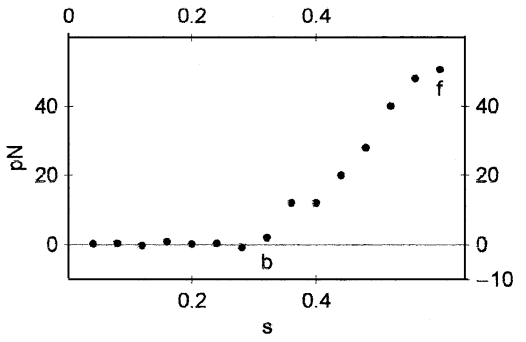

Fig. 2. Stretching a tropomyosin decorated actin filament. The alexa fluor tropomyosin decorated actin filament (n 9) was submitted to the stretching procedure. Power was 920 mW, stiffness was 0.26 pN nm^{-1}. The distance from the focus on the x-axis was recorded at 40-ms intervals. At point b, the displacement of the stage was started. At point f, the filament broke.

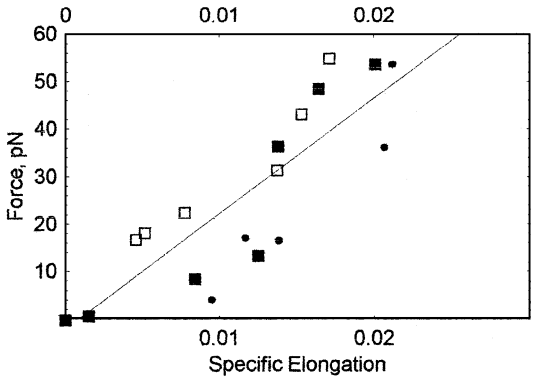

Fig. 3. Stretching force against specific elongation of filaments 2, 5 and 6. Ordinate: Force, pN; Abscissa: Specific elongation $(L-L_0)/L_0$ where L is the actual length and L_0 is the length at zero force. f2, Filled circle; f5, filled square; f6, empty square.

3. Results

3.1. Binding of alexa fluor tropomyosin to F-actin

Alexa fluor (R) 488 C_5 maleimide is a bulky reagent. It was therefore checked whether the tropomyosin labelled with 2.9 moles of alexa fluor per mole recovered the proper conformation. In a first experiment F-actin (0.45 to 48 μM, as the monomer) and alexa fluor tropomyosin were mixed at the 7:1 molar ratio and the amount of tropomyosin cosedimenting with F-actin was determined. It was found that the fraction of tropomyosin sedimenting with actin was spanning from 0.4 to 0.7, independently on the concentration of actin.

This apparently indicates that approximately half of the labelled tropomyosin binds tightly to F-actin. To estimate the binding propensity of the unbound tropomyosin, 0.33 μM alexa fluor tropomyosin was mixed with increasing concentrations of F-actin (2.41–47.6 μM as the monomer). Again the fraction of tropomyosin cosedimenting with F-actin was approximately 0.6, independent of the concentration of actin. Thus, the remaining 40% of labelled tropomyosin does not bind to actin. It was, therefore, decided to take into account the presence of the inactive tropomyosin and to perform the decoration by using 2 mol of tropomyosin per 7 mol of actin as the monomer.

3.2. The elastic modulus by stretching of the alexa fluor tropomyosin decorated actin filaments

Solutions were prepared containing 1.4 μM F-actin and 0.4 μM alexa fluor tropomyosin in 16 mM phosphate, 3 mM $MgCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol, pH 7.0. The F-actin to tropomyosin ratio was thus 7:2 to take into account the inactive fraction of tropomyosin. After 60 min of incubation at 4 °C, the samples were diluted to 20 nM (as the actin monomer) with the same buffer supplemented with the oxygen depletion system and specific elongation as a function of the stretching force applied was measured at 22 °C (Figs. 3 and 4).

For filaments 2, 5 and 6, data are described by the straight line:

$$\text{Force(pN)} = -2.046 + 2429 \times (\text{Specific Elongation}) \quad (1)$$

and for filaments 1, 3, 4, 7 and 8 by the straight line:

$$\text{Force(pN)} = 2.48 + 3916 \times (\text{Specific Elongation}) \quad (2)$$

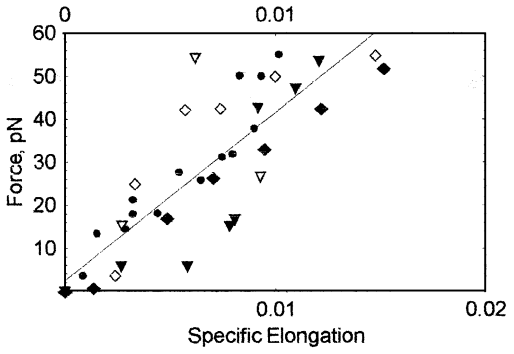

Fig. 4. Stretching force against specific elongation of filaments 1, 3, 4, 7 and 8. Ordinate: Force, pN; Abscissa: Specific elongation $(L-L_0)/L_0$ where L is the actual length and L_0, is the length at zero force. f1, Filled circle; f3, filled, upside down triangle; f4, empty, upside down triangle; f7, empty rhomb; f8, filled rhomb.

The specific elongation of the remaining eight filaments as a function of the stretching force applied is shown in Figs. 3 and 4. From these data, the elastic modulus by stretching (M) for the two sets of filaments is calculated according to the equation:

$$M = 1/(\pi.r^2).dF/ds' \quad (3)$$

where $r = 4.5$ nm is the radius of the actin filament [19]; F is the force as a function of the specific elongation and $s' = (L - L_0)/L_0$ is the specific elongation.

The elastic modulus by stretching is 38.2 and 61.48 MPa for the two sets of filaments, respectively.

3.3. Yield strength of alexa fluor tropomyosin decorated F-actin

Out of the 10 alexa flour tropomyosin decorated actin filaments, only two broke at the force of 50.5 and 55 pN, respectively, even though the laser trap was operated at the maximum power. Thus, we are able only to set the lower value of the yield strength of these filaments.

4. Discussion

This is the first time that an investigation is performed on the elastic properties of natural actin filaments, phalloidin-free and chemical modification free. The filaments were made detectable by interaction with fluorescent tropomyosin, obtained by coupling with alexa fluor (R) 488 C_5 maleimide.

When trying to determine the yield strength, even though the laser light trap was operated at its maximum power (920 mW), only two out of the 10 filaments tested were broken at 50.5 and 55 nN, respectively. We can thus set only the lowest value of the yield strength of these filaments. Based on our previous experience [6,10,11] the educated guess can be made that the yield strength interval may extend up to 70 pN as the maximum value. The yield strength of 50–55 pN is approximately five times larger than the value of 10.51 pN obtained for the yield strength of tetramethylrodamine F-actin decorated with tropomyosin, nevertheless it is still almost 1 order of magnitude lower than the putative yield strength of thin filaments in skeletal muscle [11].

Among the factors that in vivo may increase the yield strength from the ~70 pN of the alexa fluor tropomyosin F-actin to the required ~700 pN are:

1. Thin filament is embedded into the *Z* disc at one end and capped at the other end. This should decrease critical concentration and increase yield strength. Unfortunately we are unable to quantify this effect.
2. The yield strength of thin filament could be increased, let's say by a factor of two, by exposure to muscle macromolecular osmotic pressure. This is because the increase of macromolecular osmotic pressure decreases critical concentration [8] and the decrease of critical concentration increases the yield strength of the actin filament [6].
3. Decoration of the actin filament with proteins beside tropomyosin and troponin could contribute to increase the yield strength. We propose gelsolin as a candidate for this function. Gelsolin is found in skeletal muscle [20] as a part of the myofibrils [21] and particularly of the I band [22]. Gelsolin is known to fragment F-actin and to behave as a capping (barbed end) protein. It is also known, however, that fragmentation is inhibited by association of actin filaments into bundles of filaments [23]. Moreover, in myofibrils exogenous gelsolin binds to thin filaments without severing them [24]. Gelsolin is also found to displace phalloidin from the actin filament [25]. This means that binding of gelsolin to actin is very tight and it is likely to stabilise the actin filament as does phalloidin. Severing and capping are regulated by calcium concentration. Gremm and Wegner [26] showed that below 1-μM calcium ion, only the capping activity is detected. In relaxed muscle, calcium ion is ~0.1 μM and increases to ~1 μM during contraction. It also happens that, in the presence of the correct calcium concentration, binding of gelsolin to F-actin is very rapid and is followed much more slowly by the fragmentation [26]. These features make it unlikely that

gelsolin severs thin filaments in skeletal muscle and indicate, on the contrary, that it may stabilise thin filaments.

4. Furthermore, the yield strength of thin filaments could increase during contraction. In fact, when actin is decorated with myosin subfragment 1 up to the 1:1 myosin subfragment 1 to actin molar ratio, the yield strengths of tetramethylrhodamine F-actin increases from 3.69 to 15.81 pN [11]. This indicates that the yield strength of thin filament is likely to be larger in contracting than in relaxed muscle and to increase with the fraction of attached crossbridges.

At variance with the yield strength, the elastic modulus by stretching of the actin filaments decorated with alexa fluor tropomyosin (38.2–61.48 MPa) is not significantly different from that of tetramethylrodamine F-actin decorated with tropomyosin (23–75 MPa) [11]. The dissociation between yield strength and elastic modulus by stretching could indicate that elongation is mainly caused by the compliance of the bulk of the monomers of the filament and only to a minor extent by the elastic deformation of the monomer to monomer recognition surfaces.

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