Biochemical and Biophysical Research Communications **276,** 1225–1228 (2000)
doi:10.1006/bbrc.2000.3608, available online at http://www.idealibrary.com on IDEAL®

Binding of Tropomyosin–Troponin to Actin Increases Filament Bending Stiffness

Wolfgang H. Goldmann
Departments of Pathology and Surgery, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Received August 28, 2000

Rheologic measurements show that the association of tropomyosin–troponin with actin filaments is responsible for the reduction of the internal chain dynamic and increase in the mechanical rigidity of actin filaments. Basing calculations on the linear relation between the plateau modulus, G'_N, and bending modulus, κ, I find that tropomyosin–troponin at $r_{AT} = 7$ increases actin filament stiffness by ~50%. This is confirmed by dynamic light scattering. Further increases are observed at rising F-actin and constant tropomyosin–troponin concentrations. Tropomyosin–troponin also delays actin assembly and subsequent network formation and increases filament stiffness over time.

Key Words: **tropomyosin–troponin; actin filaments; stiffness; rheology; dynamic light scattering.**

Tropomyosin (Tm) and troponin (Tn) are key proteins in the regulation of muscle contraction and are found in a wide range of muscle and non-muscle cells. Tm exists as a dimer of two polypeptide subunits, which associate in register in a coiled-coil fashion and interact with actin by winding around the actin filament (1). Tn consists of three nonidentical subunits: TnT, TnI, and TnC. The interaction between TnC, TnI, and actin filaments is central to the calcium-dependent regulation of contraction and actomyosin ATPase activity (2).

In this study, I first use rheological and dynamic light scattering technique to examine the effect of tropomyosin–troponin (Tm–Tn) on actin polymerization and filament stiffness and then compare these findings with previous results. In his measurements of actin polymerization kinetics, Wegner (3) found that the rate of actin assembly is reduced in the presence of tropomyosin. This phenomenon was explained by the inhibition of spontaneous actin filament fragmentation (4). Kojima *et al.* (5) used microneedle techniques to measure the stiffness of single actin filaments in the presence of tropomyosin and reported an approximately 50% increase over that of pure actin filaments. Using quasi-elastic light scattering (QELS), Goetter *et al.* (6) investigated the effect of tropomyosin-troponin on actin filament stiffness in solution. They observed at F-actin concentrations ranging from 2.4 to 19 μM and at molar ratios of 7:1:1 (actin:Tm:Tn) a concentration independent value for the bending modulus, κ, which was ~50% higher compared to pure F-actin.

Abbreviations used: G' (ω), dynamic modulus (= elastic portion of a solution/gel); G'' (ω), loss modulus (= viscous portion of a solution/gel); G'_N, plateau modulus; $|G^*|$, absolute value of the complex shear modulus (= measure of the resistance of the solution/gel against oscillating deformation, i.e., stiffness of the gel); $J(t)$, creep (= deformation); κ, bending modulus; ω, frequency (rad/s); φ phase shift (either $\varphi \rightarrow \pi/2$ = viscous solution or $\varphi \rightarrow 0$ = elastic solution); t, time (s); α (t) rotational amplitude; r_{AT}, molar ratio of actin/tropomyosin–troponin mol/mol).

MATERIALS AND METHODS

Protein preparations. Actin was prepared (according to the procedure posted on the web site: http://iprotocol.mit.edu/protocol/307.htm and 308.htm) from acetone/ether powder obtained from rabbit back muscle, and followed by gel filtration. The biological activity of the purified actin was tested using falling ball viscometry, and its concentration was determined by measuring the absorbance, $E_{1\%,280nm} = 11.04\ cm^{-1}$ (7). Fractionated G-actin was stored in G-buffer: 0.2 mM Tris/HCl, pH 7.5; 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, and 0.005% NaN_3, for no longer than 10 days. For rheological and dynamic light scatter studies, G-actin was polymerized overnight at 4°C in F-buffer: 0.2 mM Tris/HCl, pH 7.5; 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM ATP. In experiment, where Ca^{2+} was not required, the EGTA concentration was increased to 2 mM.

The residue from the actin extraction of the acetone powder was further treated to copurify tropomyosin and troponin following the method posted on the web site: http://iprotocol.mit.edu/protocol/305.htm and /306.htm with some modifications. All buffers used contained DTT to maintain the reduced state of TnC thought to be essential for its functioning (8). The protein concentration was determined by measuring the absorbance, $E_{1\%,280nm} = 3.8\ cm^{-1}$ (9).

Rotation disc rheometer. This device has been described in detail by Mueller *et al.* (10). In brief, a glass cuvette filled with protein solution is surrounded by two perpendicularly oriented magnetic coils. One of these serves to fix the orientation of the magnetic disc on top of the solution; the other (deflection coil) applies a shear force to the viscoelastic solution. To determine the dynamic moduli $G'(\omega)$ and $G''(\omega)$, the deflection coils are driven by an oscillatory voltage at

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frequency ω. The dynamic storage and loss modulus $G'(\omega)$ and $G''(\omega)$ and the creep compliance $J(t)$ can be measured between $\omega/2\pi = 10^{-5} - 10^{1}$ Hz and $t = 10^{-1} - 10^{4}$ s, respectively. From the dynamic moduli the phase shift angle $\tan(\varphi) = G''/G'$ can be calculated (for further reading see 11–13).

Dynamic light scattering. The experimental setup for dynamic light scattering has been described in detail (6, 14). In brief, protein solutions are filled in dust-free measuring tubes of 1.5 cm diameter, which are placed in a temperature-controlled sample holder. Light from an Innova 70-4 argon-ion laser (200 mW at 488 nm) at a defined angle is detected by a photomultiplier (PM). The signal leaving the PM is amplified and filtered by a preamplifier/discriminator (PAD). The output of PAD is displayed on a counter and fed into a digital correlator. In the present experiment, the correlator ALV 3000 from ALV, Langen, Germany with 1024 linear channels was used to calculate the dynamic structure factor (for further reading see 15–17).

RESULTS AND DISCUSSION

Rheology and Dynamic Light Scattering

The goal of this study is to use rheological and dynamic light scatter methods to determine the actin filament stiffness in the presence/absence of tropomyosin–troponin. To analyze experimental results, the correlation between the plateau modulus, G'_N, and bending modulus, κ, is used, where $G'_N \sim \kappa$. Assuming that $G'_{N,T}$, κ_T, and G'_N, κ indicate the presence/absence of tropomyosin–troponin, the relation, R

$$R = G'_{N,T}/G'_N \sim \kappa_T/\kappa$$

is a direct measure of the change in actin filament flexibility induced by tropomyosin–troponin. That is, $R > 1$ would indicate an increase in filament stiffness due to binding to tropomyosin–troponin. Figure 1 shows the rheological results from creep and frequency measurements of filamentous (F)-actin in the presence of tropomyosin–troponin depicted by either the continuous lines or the symbol +, while controls (i.e., pure F-actin) are indicated by the dotted line and the symbol ◇. Both measurements were performed under identical conditions: either at 8 μM F-actin in the absence/presence of Tm–Tn at a molar ratio, $r_{AT} = 7$ (a) or at 10 μM F-actin in the absence/presence of Tm–Tn at $r_{AT} = 9$ (b). Using these molar ratios, the actin filament is assumed to be surrounded by tropomyosin molecules on both sides of the filament complexed with troponin (Fig. 2).

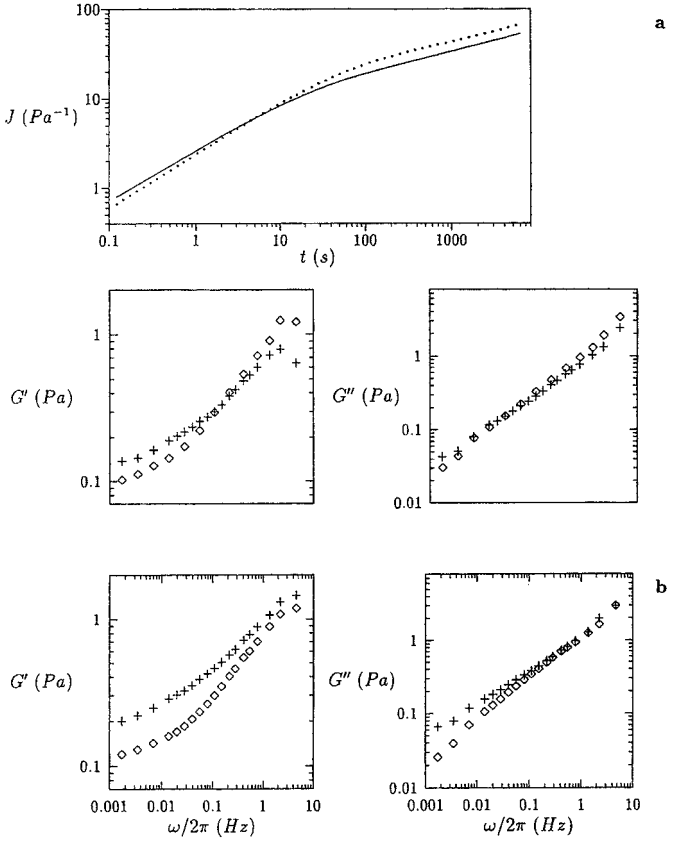

FIG. 1. Creep and frequency measurements of F-actin in the presence/absence of tropomyosin-troponin. Creep measurements using F-actin in the presence of tropomyosin–troponin are indicated by the continuous line and pure F-actin (control) are indicated by the dotted line (a). Frequency measurements at 8 μM (a) and 10 μM (b) F-actin (control) is marked by the symbol ◇, and in the presence of Tm–Tn at $r_{AT} = 7$ (a) and at $r_{AT} = 9$ (b) are indicated by the symbol +. Buffer conditions: 0.2 mM Tris/HCl, pH 7.5; 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM ATP.

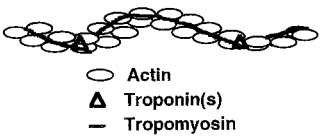

FIG. 2. A model of Tm–Tn association to actin filaments. Tm–Tn are believed to interact along the grooves of actin filaments.

Figure 1a demonstrates the effect of tropomyosin–troponin association with actin filaments in a time- and frequency-dependent manner: in the regime of the internal chain dynamic (>0.1 Hz), i.e., at short times in $J(t)$, actin filaments are more deformable with a lower elastic $G'(\omega)$ and viscous $G''(\omega)$ modulus; thus in the regime of the rubber plateau ($\omega/2\pi < 0.01$ Hz) $G'(\omega)$ exhibits a higher elastic modulus than pure actin filaments. The effect of tropomyosin–troponin association with F-actin is even more striking at higher F-actin concentration (10 μM) and lower molar ratio, $r_{AT} = 9$ (Fig. 1b). At this protein ratio, the molarities per volume are about the same as shown in Fig. 1a. The only difference exists in the 3-D structure of the actin network, which is probably more susceptible to tropomyosin–troponin binding. Comparing the frequency measurements of F-actin in the presence/absence of tropomyosin–troponin, we see that $G'(\omega)$ and $G''(\omega)$ correlate reasonably well; and in determining how the storage modulus scales with frequency, $G'(\omega) \sim \omega^{\alpha}$ in the regime of the internal chain dynamic,

TABLE 1

The Calculated *R*-Factors for F-Actin/Tropomyosin-Troponin Measurements

Sample	*R*	
	Frequency	Creep
8 μM F-actin:(Tm–Tn) at 7:1 (mol/mol)	1.49	1.53
10 μM F-actin:(Tm–Tn) at 9:1 (mol/mol)	1.87	1.47

both at $r_{AT} = 7$ and $r_{AT} = 9$ we find a reduction of α of ~25%. Note: pure F-actin scales with $\alpha = 0.45$. Based on these measurements the following molecular mechanism of tropomyosin–troponin can be assumed: (a) tropomyosin–troponin associates with actin filaments and reduces the elastic (undulation) modulus. This is indicated by the reduced frequency scaling of the storage modulus G' in the regime of the internal chain dynamic. (b) The reduced flexible behavior of individual actin filaments leads to an apparently stiffer macroscopic network. Note: the increase in network stiffness is normally observed at higher values for G' and G'' at the rubber plateau.

Table 1 shows the bending modulus of actin filaments in the presence of tropomyosin–troponin. The frequency and creep measurements were performed with different samples. The factor, *R*, for the creep was determined from the transformed spectra $J(t) \rightarrow G'(\omega)$. Special attention should be given to measurements at 8 μM actin and at $r_{AT} = 7$, where the bending stiffness of actin filaments increases by about 50%. This result agrees well with dynamic light scatter measurements (shown in Fig. 3) and with published results (6) that assume the validity of $G_N \sim \kappa$.

The mechanical changes of the actin filament network in the presence of tropomyosin–troponin could be explained by the latter's cross-linking capabilities. Tropomyosin–troponin binds at a distance of 40 nm at 7 equidistant association points (18), and one way to determine its binding would be to measure the persistence length. Compared with pure F-actin, only proteins that cause an increase in filament stiffness should increase the persistence length. Indications that tropomyosin–troponin does not have cross-linking or bundling capabilities were seen in electron micrographs (data not shown).

Actin Polymerization

The influence of tropomyosin–troponin on actin polymerization was also examined. Using 10 μM actin polymerizing in the absence (◇) and presence (+) of tropomyosin–troponin at $r_{AT} = 9$, the complex shear modulus, |G*|, and the phase shift, φ, were measured

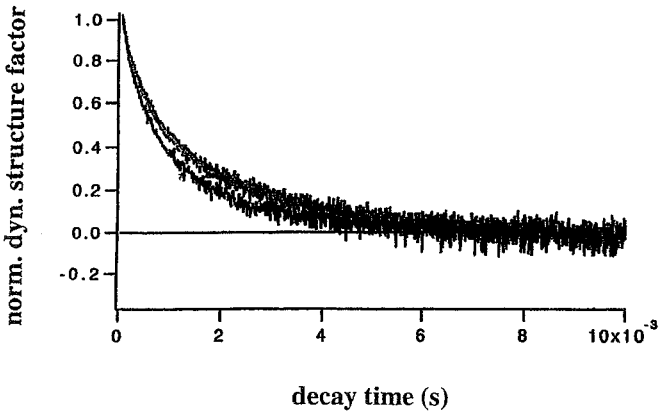

FIG. 3. The influence of tropomyosin–troponin association with 8 μM F-actin at $r_{AT} = 7$ in the presence 0.2 mM calcium measured by dynamic light scatter (upper trace) and pure actin (lower trace). Experimental conditions: scatter angle, 90°; temperature, 10°C. Buffer conditions as in Fig. 1. Data are fitted by a simple stretched exponential, $g(t) = a \times \exp(-b \times t^{0.75})$ described in (19) to obtain the dynamic structure factor, *b*. Using this analysis form, the actin filament dynamic is reduced by approximately 50% in the presence of tropomyosin–troponin compared to pure F-actin. The presence/absence of calcium showed little effect. This observation is in agreement with results by Goetter *et al.* (6).

over time (Fig. 4). In the actin polymerization curves, the presence of tropomyosin–troponin shows the following differences: (a) at $t < 800$ s $|G^*|$ and φ show a slower rate, indicating delayed actin filament network formation; (b) at $t > 800$ s higher and lower values for $|G^*|$ and φ, respectively, point to an increase in actin

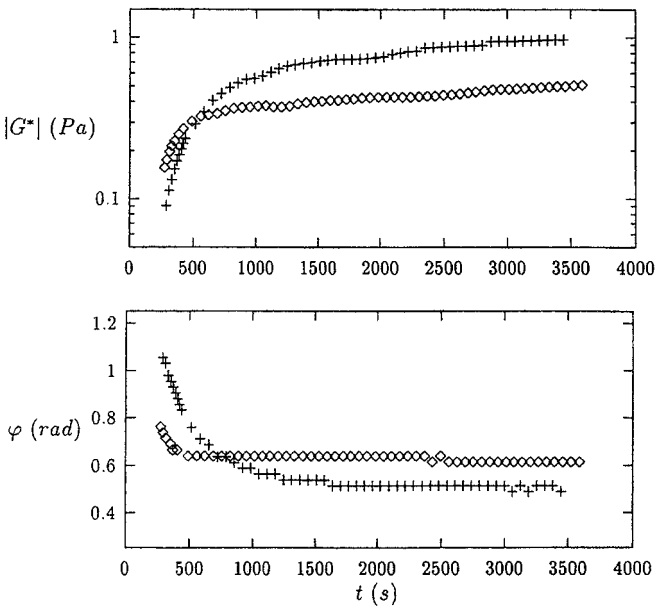

FIG. 4. Time dependence of $|G^*|$ and φ of actin polymerizing at 10 μM actin in the presence (+) and absence (◇) of tropomyosin–troponin, $r_{AT} = 9$. The measurements were performed at shear rate of 0.7 Hz. Buffer conditions as in Fig. 1.

filament rigidity. Explanations for this behavior can be found in observations made by Wegner (3), who showed that the rate of actin polymerization decreases markedly in the presence of tropomyosin but has no influence on actin nucleation. This can be explained kinetically: during pure actin polymerization, newly formed filaments spontaneously break at subunit contacts and increase the number of filament ends, which are again ready for polymerization. This behavior increases the rate of polymerization. In the presence of tropomyosin (–troponin), however, polymerization is hindered by tropomyosin's association with actin filaments. According to Wegner (4), in such cases fewer actin filament ends are available, which slows down the polymerization process. Therefore, the actin network state is reached later than it is with pure F-actin. This behavior is illustrated in Fig. 4 and confirms Wegner's hypothesis.

In summary, I present evidence using biophysical techniques that Tm–Tn significantly influences actin assembly, probably by inhibiting spontaneous fragmentation, and that its association with actin filaments dramatically reduces the internal chain dynamic by stabilizing the filaments. These observations, together with previous findings (3–6), are the basis of my ongoing research to elucidate Tm–Tn's function at the cellular level.

ACKNOWLEDGMENTS

I thank Ms. Hulda Kirpal for protein purifications, Drs. Markus Tempel and Roman Goetter for helpful discussions, and Judith Feldmann, Ph.D., for careful reading of the manuscript. W.H.G. is the recipient of a grant-in-aid from the German Government.

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