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On the elastic properties of tetramethylrhodamine F-actin

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Abstract

(Iodoacetamido)tetramethylrhodamine disrupts F-actin. At the 1:1 fluorophore to actin (as monomer) ratio approximately 80% of the protein becomes non-sedimentable. The fluorescent, non-sedimentable actin copolymerizes with G-actin to yield fluorescent filaments. The tensile strength of these filaments changes with the ratio of the fluorescent non-sedimentable actin to the G-actin, being 1.6 pN, 2.9 pN and 3.6 pN at the 1/4, 2/3 and 1/1 ratios, respectively. These tensile strengths are approximately two orders of magnitude lower than those obtained by decoration of F-actin with phalloidin.

Keywords: Rhodamine; Phalloidin; F-actin; Tensile strength

1. Introduction

The laser trap is an excellent technique which allows in vitro measurements of picoNewton forces and of nanometer steps. When applied to the study of contractile events, phalloidin F-actin (rhodamine phalloidin F-actin) is used as a substitute for the actin filament [1–7]. Unfortunately rhodamine phalloidin F-actin is a very poor substitute for native F-actin because it displays a much lower critical concentration, a higher tensile strength and different osmotic properties. The critical concentration of phalloidin F-actin is at least one order of magnitude lower than that of F-actin [8]. Critical concentration (i.e. the dissociation constant of the elongation reaction) is the main determinant of the free energy of the monomer–monomer interaction, thus of the tensile strength of the actin filament. As a matter of fact, critical concentration and tensile strength are inversely related [9], and tensile strength of phalloidin F-actin is much larger than of F-actin. Furthermore, phalloidin F-actin and F-actin display distinctly different osmotic properties [10]. It

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thus appears quite unreasonable to use phalloidin F-actin to study the mechano-elastic properties of F-actin. With the aim to overcome this drawback, we now address our attention to tetramethylrhodamine-F-actin as a possible fluorescent, bona fide substitute for F-actin. We report here that fluorescent actin filaments are obtained by copolymerization of tetramethylrhodamine-labeled, non-sedimentable actin with G-actin. Under comparable ionic strength conditions, these tetramethylrhodamine-labeled actin filaments display a tensile strength approximately two orders of magnitude lower than that displayed by rhodamine phalloidin F-actin, furthermore, their tensile strength depends on the fluorophore to actin ratio.

2. Materials and methods

Globular(G)-actin was prepared from rabbit muscle [11]. Molar concentrations were calculated on the basis of molecular masses of 42 kDa [12] and of an absorption coefficient $A^{1\%}_{290}$ of 6.2 [13].

Tetramethylrhodamine-5-iodoacetamide was purchased from Molecular Probes Europe BV, Leiden, The Netherlands. The compound was dissolved in dimethylformamide to prepare 6 mM stock solutions.

The viscosity was measured with Ostwald viscosimeters (water flow time 100 s at 20°C) maintained at 22°C.

Protein sedimentation was performed in the Beckman TL100 rotor of a 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 [14] or according to Lowry et al. [15].

2.1. Coupling F-actin with tetramethylrhodamine-5-iodoacetamide.

Before coupling 2-mercaptoethanol was removed from the F-actin solution by dialysis at 2°C against a solution containing 5 mM bicarbonate, 5 mM ascorbate, 2 mM $MgCl_2$ 0.1 M KCl 0.2 mM ATP and 2 mM NaN_3, pH 8.0. Tetramethylrhodamine-5-iodoacetamide (0.1–1 mol/mol of actin) was then added to the F-actin (2 mg/ml) solution. After 17 h of incubation at 22°C the reaction was quenched by the addition of 5 mM 2-mercaptoethanol (final concentration) and diluted to 1 mg of actin/ml with the same buffer.

Coupling was also performed at 2°C for 72 h as described by Tait and Frieden [16], with the exception that bicarbonate was substituted for Tris buffer. In this case tetramethylrhodamine-5-iodoacetamide was 0.2–2 mol/mol of actin.

The degree of labeling for rhodamine-actin was determined by making use of the extinction coefficient of 8.8 $\times$ 10^4 M^{-1} at 555 nm for the protein-bound dye.

Actin filaments were observed by means of a Zeiss microscope equipped with a Ph 3 Plan-APO CHROMAT 100 X, N.A. 1.4 objective and two PI 10 $\times$ /25 oculars. The specimens were illuminated in bright field by a 100-W halogen lamp and in epifluorescence by a 75-W Xenon-lamp. The images were captured by an RS Photometrics camera, recorded and stored on CD-ROM.

When F-actin is pre-treated with *N*-ethylmaleimide, which is known to react primarily with cysteine-374 [17], no fluorescent filaments are observed. It is therefore likely that rhodamine-actin is specifically labeled on cycteine-374.

The yield strength of the actin filaments, obtained by copolymerization of non-sedimentable tetramethylrhodamine-actin with G-actin, was determined with the apparatus and the technique previously described [9], with the exception that the measurements were performed in a solution containing 15 mM orthophosphate, 3 mM $MgCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol, pH 7.0.

3. Results

3.1. The viscosity of rhodamine F-actin

Coupling F-actin (1 mg/ml) with (iodoacetamido)tetramethylrhodamine decreases the specific viscosity of the polymer from 0.85, in the absence of the fluorophore, to either 0.82 or 0.05 M the presence of the fluorophore at the 1/10

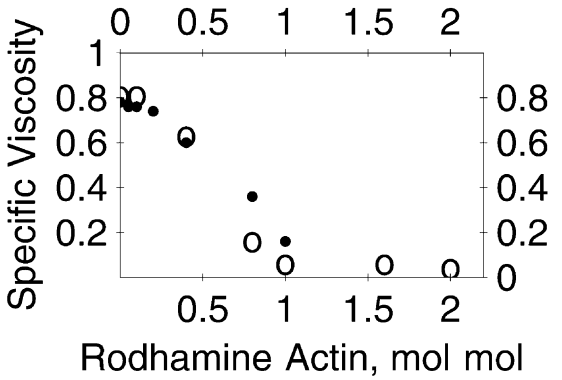

Fig. 1. Specific-viscosity of F-actin as a function of the tetramethylrhodamine-actin ratios. The incubation of tetramethylrhodamine iodoacetamide with F-actin was performed either for 17 h at 22°C ● or for 72 h at 2°C ○.

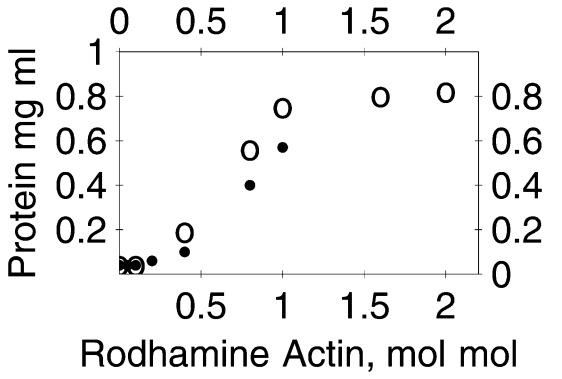

Fig. 2. Mass of non-sedimentable actin as a function of the tetramethylrhodamine to actin ratios. The incubation of tetramethylrhodamine iodoacetamide with F-actin was performed either for 17 h at 22°C ● or for 72 h at 2°C ○.

and 2/1 fluorophore to actin (as monomer) ratios, respectively. Essentially the same results are obtained by labeling F-actin either for 17 h at 22°C or for 72 it at 2°C (Fig. 1).

3.2. The sedimentation of rhodamine F-actin

Sedimentation of F-actin (1 mg/ml) in the absence of tetramethylrhodamine-iodoacetamide yields supernatant solutions containing, on average, 0.04 mg of protein/ml. Coupling with (iodoacetamido)tetramethylrhodamine increases protein in the supernatant solution: these become either 0.1–0.2 mg/ml or 0.6–0.7 mg/ml or 0.8 mg/ml at the 4/10, 1/1 and 2/1 fluorophore to actin ratios, respectively (Fig. 2).

3.3. Fluorescence microscope observation of the tetramethylrhodamine-labeled actin filaments

Labeled actin was diluted to 20 nM (as the monomer) with a solution containg 15 mM orthophosphate, 3 mM $MgCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol, pH 7.0. Fluorescent actin filaments were clearly detected in the samples labeled at the 1/5 to the 0.8/1 fluorophore to actin ratios. The actin filaments labeled at the 2/5 fluorophore to actin ratio seemed to be shorter on average than those labeled at the 1/5 fluorophore to actin ratio.

The effect of labeling on the length of the filaments is also deduced from the upward concavity of the plot of the normalized specific-viscosity against the normalized sedimentable protein concentration (Fig. 3). The upward con-

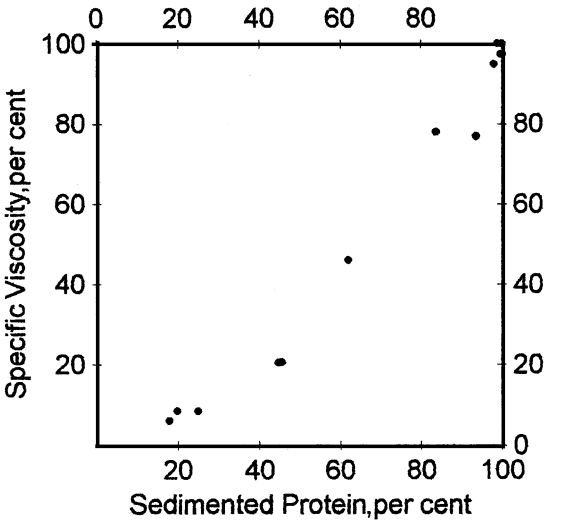

Fig. 3. Tetramethylrodamine labeled actins: specific- viscosity against sedimentable protein. Ordinate: 100 × specific viscosity of the labeled actin samples/specific viscosity of the unlabeled actin sample; abscissa: 100 × (sedimentable actin, mg/ml,-critical concentration of unlabeled actin, mg/ml)/(total actin, mg/ml, critical concentration of unlabeled actin, mg/ml unlabeled actin: upper, right corner; labeled actin (2/1 rhodamine to actin ratio): lower, left corner. Data are taken from Figs. 1 and 2.

cavity indicates that, with the increase of the rhodamine/actin ratio, the decrease of the specific-viscosity is larger than the decrease of the sedimentable protein, thus the average filament length decreases.

3.4. The state of aggregation of actin in the supernatant solutions

To investigate the state of aggregation of actin in the supernatant solutions obtained by centrifugation of F-actin labeled with tetramethylrhodamine at the 1/1 fluorophore to actin ratio, their light scattering at 600 nm (90° observation angle) was compared with that of freshly centrifuged G-actin solutions. As shown in Table 1 the light scattering of the supernatant solution was, on a molar basis, approximately fivefold larger than that of G-actin, thus showing the presence of small oligomers in the solution.

3.5. Copolymerization of G-actin with tetramethylrhodamine-actin

The supernatant solutions, obtained by centrifugation of F-actin labeled with tetramethylrhodamine at the 1/1 fluorophore to actin ratio, were tested for their capability to copolymerize with G-actin. To do this, labeled actin (0.75 mg/ml) and unlabeled G-actin (2 mg/ml) were mixed in different proportions, in the presence of 100 mM KCl and 2 mM MgCl, (final concentrations), to yield solutions (total actin 1 mg/ml), containing: 0, 10, 20, 30, 40 and 50% labeled actin per total actin, respectively. After 1 h of incubation at 25°C, the mixtures were diluted to 20 nM actin (as the monomer) with a solution containg 15 mM orthophosphate, 3 mM $MgCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol and were observed at the fluorescence microscope. Fluorescent filaments were detected in the samples containing 20–50% labeled actin per total actin.

The copolymerization products were also tested for viscosity and for sedimentation. Specific-viscosity was found to decrease from 0.98 to 0.53 and protein in the supernatant solutions was found to increase from 0.04 to 0. 19 mg/ml when tetramethylrhodamine-actin was increased from 0 to 50% of total actin (Table 2). Since added tetramethylrhodamine-actin is non-sedimentable, per se the increase of the non-sedimentable protein does not necessarily indicate that the critical concentration of the newly formed, fluorescent F-actin increases. To ascertain this point the product obtained by the copolymerization of 1 mol of G-actin with 1 mol of non-sedimentable tetratmethylrhodamine-actin was diluted serially with the polymerization buffer, left 17 h at 22°C and centrifuged for 10 min at 360 000 × g. Protein concentration in the supernatant solution was 0.131, 0.094, 0.058, 0.035 mg/ml, as compared to a total protein concentration of 0.5, 0.25, 0.125 and 0.062 mg/ml, respectively. As a conclusion the apparent critical concentration (0.035 mg/ml) was close to that of F-actin.

3.6. The degree of labeling of the copolymerized filaments

The degree of labeling of the filaments obtained by copolymerization (1/1 by weight) of the labeled supernatant solution (degree of labeling 0.53) with unlabeled G-actin was determined on

Table 1
Light scattering of G-actin and of the supernatant solutions obtained by centrifugation of F-actin labeled with tetramethylrhodamine at the 1/1 fluorophore to actin ratio

	Light scattenng (a.u.)
G-actin 24 μM	105
Supernatant solution 16.8 μM	362

F-actin (24 μM as the monomer) was labeled with tetramethylrhodamine-iodoacetamide at the 1/1 fluorophore to actin ratio in 5 mM bicarbonate, 5 mM ascorbate, 2 mM $MgCl_2$, 0.1 M KCl, 0.2 mM ATP and 2 mM NaN_3, pH 8.0. After 17 h of incubation at 22°C the sample was dialysed against the same buffer to remove unbound fluorophore. After dialysis the sample was centrifuged for 10 min at 360 000 × g and the light scattering (90° observation angle) of the supernatant solutions (16.8 μM as the monomer) was tested at 600 nm G-actin (24 μM as the monomer) in 2 mM Tris–HCI, 2 mM 2-mercaptoethanol, 0.2 mM ATP, 0.2 mM CaCl and 2 mM NaN_3, pH 8.0 was centrifuged for 10 min at 360 000 × g and the light scattering (90° observation angle) was tested at 600 nm.

Table 2
Sedimentation, viscosity and yield strength of the products of the copolymenzation of G-actin with tetramethylrhodamine-labeled, non--sedimentable actin

TMR-actin (mg/ml)	G-actin (mg/ml)	Total protein (mg/ml)	Protein in the supernatant (mg/ml)	Specific-viscosity	Yield strength (pN)
0.0	1.0	1.05	0.04	0.98	–
0.1	0.9	1.04	0.09	0.82	–
0.2	0.8	1.04	0.11	0.63	1.6 ± 0.14
0.3	0.7	0.96	0.12	0.62	–
0.4	0.6	0.90	0.14	0.57	2.9 ± 0.14
0.5	0.5	0.93	0.19	0.53	3.6 ± 0.24

Tetramethylrhodamine-labeled, non-sedimentable actin (0.75 mg/ml) and unlabeled G-actin (2 mg/ml) were mixed in different proportions, in the presence of 100 mM KCl and 2 mM $MgCl_2$ (final concentrations), to yield the solutions indicated in the Table. After 1 h of incubation at 25°C, the solutions were tested for the viscosity and sedimentability as described in the Methods section. The yield strength was tested as described in the Methods section. Data represent the average of 4, 5 and 8 filaments from the samples obtained by copolymerization of 20, 40 and 50% fluorescent actin over total actin, respectively.

the pellets obtained by centrifugation for 10 min at $360\,000 \times g$. The resulting degree of labeling was 0.32 instead of the expected 0.265. This shows that in the course of the copolymerization the filament is enriched with the labeled component.

3.7. The effect of mixing non-sedimentable tetramethylrhodamine-actin with F-actin

The experiments described in the previous section were repeated by substituting unlabeled F-actin for unlabeled G-actin. In no case fluorescent actin filaments were detected. This shows that the fluorescent filaments obtained by mixing tetramethylrhodamine-labeled actin with G-actin are indeed the products of a copolymerization and not the products of the decoration of F-actin by tetramethylrhodamine-actin.

3.8. Measuring the yield strength of the fluorescent actin filaments obtained by copolymeryzation of tetramethylrhodamine-actin with G-actin

The yield strength of the fluorescent filaments obtained by the copolymerization of tetramethylrhodamine-actin with G-actin was tested in the laser trap. Filaments were diluted to 20 nM (as the actin monomer) in 15 mM orthophosphate, 3 mM $MgCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol. As shown in Table 2, the yield strength increased with the increase of the fluorescent component, being 1.6 ± 0.14 pN, 2.9 ± 0.14 pN and 3.6 ± 0.24 pN at 20, 40 and 50% fluorescent actin over total actin, respectively (Table 2).

4. Discussion

Treatment with (iodoacetamido)tetramethylrhodamine disrupts the actin filament. This phenomenon- was never mentioned specifically for rhodamine, however, modification at the actin C-terminus by substitution or truncation was previously reported to affect filament stability [18,19]. Apparently the yield, as polymeric actin, of the F-actin labeled with tetramethylrhodamine iodoacetamide was never tested [16,20–23]. The disruption of the polymer increases with the increase of (iodoacetamido)tetramethylrhodamine. Up to 80% of the protein becomes non-sedimentable when (iodoacetamido)tetramethylrhodamine, 48 μM, is added to F-actin, 48 μM, as the monomer. The length of the filaments surviving the treatment with (iodoacetamido)tetramethylrhodamine seems to shorten with the increase of the fluorophore to actin ratio. This is deduced both from direct observation and from the up-

ward concavity of the plot of the normalized specific viscosity against the normalized sedimentable protein concentration.

Surprisingly non-sedimentable tetramethylrhodamine-actin copolymerizes with G-actin to yield fluorescent actin filaments. The process is truly a copolymerization. In fact mixing non-sedimentable tetramethylrhodamine-actin with preformed F-actin does not yield fluorescent actin filaments. Copolymerization was performed at different G-actin to non-sedimentable tetramethylrhodamine-actin ratios. When this ratio was 1/1, the apparent critical concentration of the fluorescent, copolymerized F-actin was practically the same as that of non-modified F-actin. We refer to an apparent critical concentration because in our opinion G-actin is polymerizing on seeds of non-sedimentable tetramethylrhodamine-actin oligomers.

The yield strength of the fluorescent, copolymerized filaments increases from 1.6 to 3.6 pN when the original tetramethylrhodamine-actin to G-actin ratio increases from 1/4 to 1/1. This indicates that the mechanic properties of the actin filament are influenced by the labeling.

At the physiological ionic strength the Yanagida group reported that the tensile strengths of phalloidin F-actin is spanning from ~ 100 pN [8] to ~ 400 pN [24]. We reported that the tensile strength of tetramethylrhodamine phalloidin F-actin decreases significantly with the decrease of the ionic strength, being 28.6 pN and 3 pN at the ionic strengths of 19 and 3 mMg respectively [9]. At a ionic strength close to the physiological one, we find now that the tensile strength of tetramethylrhodamine F-actin spans from 1.6 to 3.6 pN, depending on the labeling ratio. The importance of this observation is two fold. First, it shows that phalloidin (1/1 phalloidin to actin ratio) alters significantly (approx. two orders of magnitude) the tensile strength, thus the mechanics of F-actin. Second, it shows that the direct coupling with (iodoacetamido)tetramethylrhodamine also influences the tensile strength, thus the mechanics of the polymer. It is thus clear that neither rhodamine phalloidin F-actin nor rhodamine F-actin are identical to F-actin.

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