Mechanical Properties of Actin Filament Networks Depend on Preparation, Polymerization Conditions, and Storage of Actin Monomers

Jingyuan Xu,* William H. Schwarz,# Josef A. Käs,§ Thomas P. Stossel,§ Paul A. Janmey,§ and Thomas D. Pollard¶

*Department of Biophysics and Biophysical Chemistry, #Department of Chemical Engineering, and ¶Department of Cell Biology and Anatomy, School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205, and §Division of Experimental Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115 USA

ABSTRACT This study investigates possible sources for the variance of more than two orders of magnitude in the published values for the shear moduli of purified actin filaments. Two types of forced oscillatory rheometers used in some of our previous work agree within a factor of three for identical samples. Polymers assembled in EGTA and Mg^{2+} from fresh, gel-filtered ATP-actin at 1 mg/ml typically have an elastic storage modulus (G′) of ~1 Pa at a deformation frequency of 0.1–1 Hz. G′ is slightly higher when actin is polymerized in KCl with Ca^{2+} and Mg^{2+}. Gel filtration removes minor contaminants from actin but has little effect on G′ for most preparations of actin from acetone powder. Storage of actin monomers without frequent changes of buffer containing fresh ATP and dithiothreitol can result in changes that increase the G′ of filaments by more than a factor of 10. Frozen storage can preserve the properties of monomeric actin, but care is necessary to prevent protein denaturation or aggregation due to freezing or thawing.

INTRODUCTION

Actin filaments, intermediate filaments, and microtubules, individually and in association with each other, determine the elastic properties of cytoplasm. Measurements of the elastic resistance of cells such as activated platelets and endothelial cells suggest that their mechanical properties resemble gels with shear moduli on the order of 100-1000 Pa. These elastic moduli allow cells to maintain their shape when exposed to shear stresses of this magnitude exerted by their active contractile machinery or by fluid flow in blood vessels (Evans et al., 1993; Oliver et al., 1994). Changes in cell shape presumably result from variations in cytoskeletal architecture, and actin polymers are major components of cytoskeletal networks in these and other cell types.

Actin filament networks are viscoelastic; that is, they both store and dissipate mechanical energy. For oscillatory deformations, the viscoelasticity is characterized by dynamic elasticity G′ (also called dynamic storage modulus) and loss shear modulus G″, which are the ratios of stress (force/area) in and out of phase, respectively, to an imposed oscillating strain (deformation). A highly elastic response of a polymer solution is characterized by $G' \gg G''$.

In contrast to the consistent data on densely cross-linked actin gels (Janmey et al., 1990a; Maciver et al., 1991; Wachsstock et al., 1993, 1994), previous measurements of the viscoelastic properties of filaments of purified actin alone have failed to provide a consistent picture of actin rheology. Different laboratories, measuring apparently similar actin preparations at a concentration of ~1 mg/ml have reported shear moduli from 0.01 Pa to several tens of Pa (Table 1). Lacking a rigorous theory to relate the macroscopic viscoelasticity of networks composed of un-cross-linked, worm-like actin polymers to their well characterized microscopic flexibility, there has been no firm theoretical basis to evaluate these widely discrepant measurements. Recent progress in theoretical modeling of semiflexible polymers suggests that quantitative solutions might soon be available and may help evaluate the experimental results (MacKintosh et al., 1995; Isambert and Maggs, 1996; Satcher and Dewey, 1997; Kroy and Frey, 1996; Maggs, 1997). On the other hand, definitive experimental characterization of purified actin is necessary to establish the validity of these models and to evaluate how various actin-binding proteins alter actin rheology and influence cellular mechanics.

Two groups that previously reported very different values for viscoelastic parameters of purified actin filaments have collaborated to explore the basis for these discrepancies. In addition to previously proposed explanations based on mechanical perturbations during rheological testing or on unrecognized contamination of actin preparations with factors that alter filament length or introduce cross-links between filaments, we also considered how differences in preparation, polymerization, and storage of actin influence its rheology. When comparing different freshly purified samples of 1 mg/ml actin filaments using different machines that impose small strain oscillations of approximately 1 Hz, shear moduli were in the range of 0.5–2 Pa for Mg-ATP actin filaments. Within this range, the methods used to

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Address reprint requests to Dr. Thomas D. Pollard, Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, CA 92037. Tel: 619-453-4100; Fax: 619-546-0838; E-mail: pollard@salk.edu.

Dr. Xu's current address: Department of Chemical Engineering, Johns Hopkins University.

Dr. Käs's current address: Physics Department and Center for Nonlinear Dynamics and Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas 78712.

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TABLE 1 Reports of rheological parameters for purified actin filaments

Purification	Ligands	Concentration (mg/ml)	Frequency (Hz)	G′ (Pa)	G″ (Pa)	|G*| (Pa)	Reference
Muscle, cycled	CaATP	2.0	0.02–0.35	0.1–0.2	0.05–0.14		Maruyama et al., 1974
Muscle, GF	CaATP	1.7	0.08–1.6	1–4	0.5–4.5		Jen et al., 1982
Muscle, cycled	CaATP	1.0	0.004–0.09			0.06–0.13	Zaner and Stossel, 1982
Muscle, GF	CaATP	1.0	0.004–0.09			0.08–0.22	Zaner and Stossel, 1982
Muscle, GF	CaATP	1.0	0.005–0.1	0.06–0.1	0.02–0.04		Zaner and Stossel, 1983
Liver, cycled	CaATP	0.8	0.001–0.1	0.012–0.03	0.01–0.03		Oppermann and Jaberg, 1985
Amoeba, GF	CaATP	1.0	0.0002–1.0	1.0–2.0	0.2 –1.0		Sato et al., 1985
Muscle, GF	CaATP	1.0	0.0002–1.0	0.4–1.0	0.1–0.9		Sato et al., 1985
Muscle, GF	CaATP	1.0	0.005–0.5	0.08–0.2	0.03–0.3		Zaner, 1986
Muscle, GF	CaATP	1.0	0.00006–1	0.7–1.0	0.03–0.3		Zaner and Hartwig, 1988
Muscle, cycled	CaATP	0.5		20			Janmey et al., 1988
Muscle, cycled	CaATP	1.4+G	0.005–1.6	12–15	0.04–0.08		Janmey et al., 1990b
Muscle, cycled	CaADP	1.6+G	0.005–1.6	92–100	1.3–4.0		Janmey et al., 1990b
Muscle, cycled	?	0.7	0.16	40	0.06		Hvidt and Heller, 1990
Muscle, cycled	CaATP	2.0	0.16	110–500			Hvidt and Janmey, 1990
Muscle, cycled	CaATP	1.3		60			Janmey et al., 1991
Muscle, cycled	CaATP	1.5		100			Janmey et al., 1991
Muscle, cycled	CaATP	2.0		283			Janmey et al., 1991
Muscle, GF	MgATP	0.4	0.0002–1.0	10–20			Maciver et al., 1991
Muscle, GF	MgATP	0.4	0.0002–1.0	0.1–0.2			Maciver et al., 1991
Muscle, GF	?ATP	0.6	0.004	3.4			Muller et al., 1991
Muscle, GF	MgADP	1.4	0.0002–1.0	0.7–2.0	0.1–0.7		Pollard et al., 1992
Muscle, GF	MgATP	1.4	0.0002–1.0	0.7–2.0	0.1–0.7		Pollard et al., 1992
Muscle, GF	MgATP	0.3	0.0001–1.0	0.01–0.2	0.01–1.0		Ruddies et al., 1993
Muscle, cycled	CaADP	1.6+G	0.0016–1.6	0.2–0.4	0.04–0.3		Newman et al., 1993
Muscle, cycled	CaATP	1.4+G	0.0016–1.6	0.1–0.3	0.04–0.3		Newman et al., 1993
Muscle, GF	MgATP	0.6	0.0002–1.0			0.2–1.0	Wachsstock et al., 1993
Muscle, cycled	CaATP	1.4	0.004–16	>100	0.7–4.0		Janmey et al., 1994
Muscle, GF	MgATP	0.6	0.0002–1.0			0.2–1.0	Wachsstock et al., 1994
Muscle, GF	MgATP	0.3	0.09–3.0	0.02–0.05	0.008–0.07		Ziemann et al., 1994
Muscle, GF	MgATP	1.0	0.2	7–15	1.4		Janssen et al., 1996
Muscle, GF	CaATP	0.4	0.0004	0.5			Tempel et al., 1996
Muscle, cycled	CaATP	0.7	1.6	6.0	0.8		Allen et al., 1996
Muscle, GF	CaATP	0.6	1.6	23	3.0		Allen et al., 1996
Aorta, cycled	CaATP	1.0	1.6	1.6	0.7		Allen et al., 1996
Gizzard, cycled	CaATP	0.6	1.6	1.0	0.2		Allen et al., 1996

Muscle, skeletal muscle actin; cycled, purified by cycles of polymerization and depolymerization; GF, cycled and further purified by gel filtration; ligands, tightly bound divalent cation and nucleotide on the monomers used for polymerization; concentration, concentration of polymerized actin; G, 1:1000 gelsolin to actin monomers.

prepare and polymerize actin monomers and the machines used for the measurements each contribute small differences in the observed values. After storage of actin monomers under some conditions, 1 mg/ml actin filaments have an elastic modulus >10 Pa. Storage in fresh buffer avoids these changes.

MATERIALS AND METHODS

Actin purification

We used two slightly different methods to purify actin from rabbit skeletal muscle. Acetone powders of rabbit skeletal muscle used for preparation A (PrepA) were prepared according to the original method of Straub (1943) as modified by Adelstein et al. (1963). The 1989 acetone powder had been stored for 2–3 years at the time of the experiments. The 1992, 1993, 1994, and 1995 acetone powders were used within a few months of preparation. The acetone powder used for preparation B (PrepB) was prepared by the method of Eichinger et al. (1991). The acetone powders were stored at −20°C in capped plastic tubes.

PrepA followed the method of MacLean-Fletcher and Pollard (1980), but using Sephacryl S-300 instead of Sephadex G-150 for gel filtration. This method is a modification of the classic method of Spudich and Watt (1971). Each gram of acetone powder was extracted for 30 min on ice with 20 ml of buffer G1 (2 mM Tris-Cl, 0.2 mM ATP, 0.5 mM dithiothreitol (DTT), 0.1 mM $CaCl_2$, 1 mM sodium azide, pH 8.0, at 25°C). Insoluble material was pelleted by centrifugation at 16,000 rpm at 2°C for 30 min, and the pellet was resuspended in the same volume of buffer G1 and immediately centrifuged as before. The supernatants were combined, and actin was polymerized by the addition of $MgCl_2$ to 2 mM and KCl to 50 mM at 4°C. After 1 hour, KCl was added to 0.8 M, and the actin filaments were pelleted by centrifugation at 100,000 × *g* for 2 h at 4°C. The filaments were depolymerized by dialysis against four 1-L changes of buffer G1 over 2 days at 4°C, and any remaining filaments were pelleted by ultracentrifugation. The top two-thirds of the supernatant was gel filtered on a 2.5 × 110 cm column of Sephacryl S-300 equilibrated with buffer G1 to remove minor contaminants that can alter the viscosity of polymerized actin (MacLean-Fletcher and Pollard, 1980). Fractions of 4 ml were collected. The peak and following fractions were pooled and used for experiments. Purified monomeric actin was stored at 4°C in four different ways: 1) in a 15-ml plastic tube with a screw cap, 2) in a dialysis bag

equilibrated with fresh buffer G1 changed daily, 3) in a dialysis bag equilibrated versus buffer G1 but not changed, 4) in a dialysis bag equilibrated with deionized water.

In PrepB, each gram of acetone powder was extracted once for 30 min at 4°C with 20 ml of buffer G2 containing 2 mM Tris-Cl, 0.5 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, pH 8.0 (at 25°C). Insoluble material was removed from the extract by filtration through cheese cloth, rinsing the residue with 5% of the total volume of buffer G2, and centrifugation at 10,000 rpm for 30 min at 4°C. The monomers were polymerized by addition of 50 mM KCl and 2 mM $MgCl_2$. After 2 hours at 25°C, KCl was added to 0.8 M, and the actin filaments pelleted by centrifugation at 100,000 $\times$ *g* for 2 h at 4°C. After depolymerization of the filaments by dialysis against buffer G2, remaining filaments were pelleted at 100,000 $\times$ *g*, and the actin monomers in the supernatant were stored frozen at −80°C after freezing in small volumes (0.1–0.3 ml) in liquid nitrogen. These samples were thawed rapidly with gentle agitation under running water at 37°C.

Rheometry

We used two types of commercial rheometers to characterize the mechanical properties of polymerized actin solutions. Sato et al. (1985) described the use of the cone and plate R18 Weissenberg rheogoniometer (Sangamo Controls, Bognor Regis, England) in the small-amplitude (the strain was less than 2%), forced oscillation mode. A solution of actin monomers was mixed with concentrated polymerizing buffer and immediately placed between the stainless steel plates at 25°C. The plates were sealed with mineral oil (Sigma Chemical Co., St. Louis, MO) to prevent sample dehydration. After at least 8 h of equilibration at 25°C, multiple readings were taken at 0.1896 Hz to ensure that the sample did not change during the experiment, which lasted up to 8 h. The second type of instrument was a Rheometrics RFS II (Rheometrics Scientific, Piscataway, NJ) rheometer. The machine in Boston was equipped with a cone and plate (Janmey et al., 1994), and the one at the University of Maryland Department of Chemical Engineering had parallel plates. Actin was polymerized between the stainless steel plates, and the plates were sealed with mineral oil. We compared two concentrated polymerization buffers, which were added to 9 vol of actin in buffer G1 or G2. The 10X KME8 contained 500 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, 20 mM Tris, pH 8.0. The 10X KMC7.4 contained 20 mM $MgCl_2$, 1500 mM KCl, 20 mM Tris, pH 7.4.

Stokes' radii and polymer lengths

Stokes' radii were determined by gel filtration at 4°C in buffer G1 on a 2.5 $\times$ 110 cm column of Sephacryl S-300 calibrated by the method of Siegel and Monty (1966) with myoglobin, catalase, bovine serum albumin, and blue dextran (Sigma). The length distributions of actin filaments were measured by fluorescence microscopy after labeling with rhodamine-phalloidin and adsorption to a glass coverslip (Xu et al., submitted for publication). This method is valuable for comparisons but is limited to some extent because ~10% of long filaments break during adsorption and filaments shorter than 0.5 μm appear as points of light and are grouped together when measuring linear dimensions.

RESULTS

This collaboration sought to identify variables that account for differences that our groups have reported for the rheological properties of filaments of purified actin. We take these variables one at a time.

Rheometers

Over a wide range of frequencies the Rheometrics RFS II rheometer in Boston recorded values for dynamic shear moduli that were two- to threefold higher for identically prepared actin filament samples than the Weissenberg rheometer in Baltimore and the Rheometrics RFS II rheometer at the University of Maryland (Fig. 1 and Table 2). In the latter two instruments, the elastic modulus (G′) was 0.7 Pa with a standard deviation of 0.1 Pa at a frequency of 0.1–0.2 Hz for 1 mg/ml PrepA actin polymerized in Mg/EGTA buffer with 50 mM KCl. All three rheometers are strain controlled. With strain set to <2%, actin filament networks were not destroyed or oriented during the measurements. Polymerization of actin monomers between the rheometer plates avoided pre-shearing and alignment of the filaments.

Polymerization buffers

In previous studies, our laboratories polymerized actin in different buffers, assuming that fully polymerized actin would have a characteristic elastic modulus. Starting with Ca-ATP-actin monomers, both polymerization buffers fully polymerize actin (critical concentrations ~0.1 μM; Pollard and Cooper, 1986), but the subunits in the filaments have different cations bound at the high-affinity site. In low Ca^{2+}, KME8 buffer containing EGTA and $MgCl_2$, Ca^{2+} bound to monomers exchanges for Mg^{2+} during the lag phase of polymerization, yielding largely Mg-actin filaments. Polymerization in KMC7.4 buffer containing both Ca^{2+} and Mg^{2+} produces Ca-actin filaments, due to higher affinity of ATP-actin monomers for Ca^{2+} (Estes et al. 1992; Gershman et al., 1994) and slow exchange of divalent cations bound to filaments.

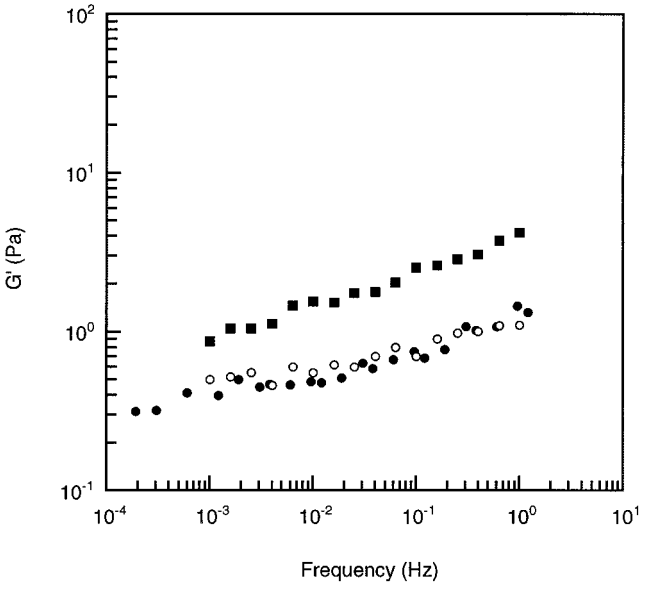

FIGURE 1 Mechanical properties of actin filaments measured with different rheometers. PrepA actin was polymerized at 1 mg/ml with concentrated polymerization buffer with final concentrations of 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM Tris (pH 8.0). ●, G′ measured with a Weissenberg rheometer; ○, G′ measured with a Rheometrics RFS II rheometer at University of Maryland (College Park); ■, G′ measured with a Rheometrics RFS II rheometer at Harvard Medical School.

TABLE 2 Summary of rheological measurements on purified actin preparations

Rheometer (number)	Concentration (mg/ml)	Powder	Purification	Buffer	Frequency (Hz)	G′ (Pa)	G″ (Pa)
Weissenberg							
12	1.0	A	PrepA, GF	KME8	0.0002–1.2	0.2–1.2	0.08–1
Average value					0.19	0.7 ± 0.11	0.23 ± 0.03
	1.0	A	PrepA not GF	KME8	0.0002–1.2	0.1–1.1	
	1.0	A	PrepA, GF, frozen*	KME8	0.0002–1.2	0.3–1.5	0.05–1
	1.0	A	PrepA, GF, frozen*	KME8	0.0002–1.2	20–25	4.0–5.0
	1.0	A	PrepA, GF, frozen*	KME8	0.0002–1.2	18–24	3.0–5.5
	1.0	E	PrepB, frozen#	KME8	0.0002–0.4	10–30	2–10
	1.0	E	PrepA, GF, fresh	KME8	0.0002–1.2	0.4–1.0	
	1.0	E	PrepA, GF, 5 days	KME8	0.0002–1.2	60–80	
Rheometrics (Boston)							
	1.0	A	PrepA, GF	KME8	0.0025–1	0.8–3.5	0.1–2.8
	1.0	A	PrepA, GF	KMC7.4	0.0025–1	1.3–11	0.4–10
	1.0	A	PrepA, GF	KME8	0.16	2.5	1.2
	1.0	A	PrepA, GF	KMC7.4	0.16	2.9	1.1
	1.5	A	PrepA, GF	KME8	0.16	1.4	0.6
	1.5	A	PrepA, GF	KMC7.4	0.16	1.9	0.9
	1.9	A	PrepA, GF	KME8	0.0025–1	0.9–2.8	0.05–1.3
	2.0	A	PrepA, GF	KME8	0.16	1.0	0.4
	2.0	A	PrepA, GF	KMC7.4	0.16	1.8	0.75
	2.0	E	PrepB, frozen#	KME8	0.16	3.3	0.8
	2.0	E	PrepB, frozen#	KMC7.4	0.16	11	2.5
Rheometrics (Maryland)							
8	0.63	A	PrepA, gel filtered	KME8	0.0002–1.6	0.2–1.0	0.05–0.7
Average values					0.16	0.56 ± 0.12	0.20 ± 0.04
7	1.0	A	PrepA, gel filtered	KME8	0.0002–1.6	0.3–1.2	0.08–0.9
Average values					0.16	0.69 ± 0.11	0.25 ± 0.04
7	1.0	A	PrepA, gel filtered	KMC7.4	0.0002–1.6	0.3–1.7	0.08–0.9
Average values					0.16	0.85 ± 0.2	0.21 ± 0.05

A, Adelstein acetone powder; E, Eichinger acetone powder. Errors are ±1 SD of the mean.
*Frozen in 1-ml aliquots.
#Frozen in 50-μl droplets.

The average elastic modulus (G′) of Ca-actin filaments was 23% higher than Mg-actin filaments (Table 2). This difference might be related to faster polymerization in KME8 buffer (half-time, 20 s) than KMC7.4 buffer (half-time, 100 s). More rapid nucleation in KME8 should, in principle, produce a larger number of short filaments. However, when measured by fluorescence microscopy after reaction with rhodamine-phalloidin and dilution to a concentration of a few nanomolar, the length distributions of both preparations were exponential with the same average lengths: 5.5 μm in KME8 and 5.6 μm in KMC7.4.

Actin preparation and storage

In direct comparisons at two concentrations and in both polymerization buffers, the shear moduli of filaments assembled from actin prepared by different methods were not the same. The shear moduli of filaments of PrepB actin were higher by 2- to 30-fold than filaments of fresh, gel-filtered PrepA actin (Table 2). Similar differences were obtained in the Rheometrics rheometer in Boston (Table 2) and the Weissenberg rheometer (Fig. 2). Systematic comparison of the preparation and storage methods revealed that none of the individual methodological differences can account for this difference in rheological properties.

Acetone powders

The two laboratories prepare the starting material for actin purification, an acetone powder of rabbit skeletal muscle, slightly differently. However, fresh PrepA actin from both acetone powders had G′ $<$ 1 Pa in both types of rheometers (Table 2; Fig. 2).

Purification

Both preparations start with a low-salt extraction of the acetone powder, followed by one cycle of polymerization and depolymerization and then ultracentrifugation to remove residual filaments. PrepA actin is further purified by gel filtration to remove actin oligomers and minor contaminants that can increase or decrease the viscosity when added back to purified actin (MacLean-Fletcher and Pollard, 1980). Although gel filtration improves the purity of

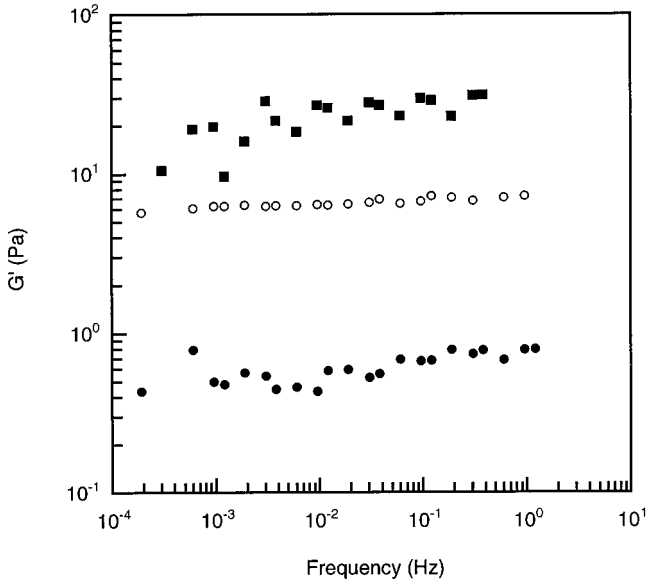

FIGURE 2 Comparison of G′ of actin filaments polymerized from the two types of actin preparations. PrepA actin was purified from an acetone powder made by the method of Eichinger (1991) and stored in a capped plastic tube at 4°C for 1 day (●) or 5 days (○). PrepB actin was purified from the same acetone powder in Boston, stored frozen, and transported to Baltimore on ice in a capped plastic tube (■). Actin was polymerized at l mg/ml in KME8, and G′ was measured in the Weissenberg rheometer.

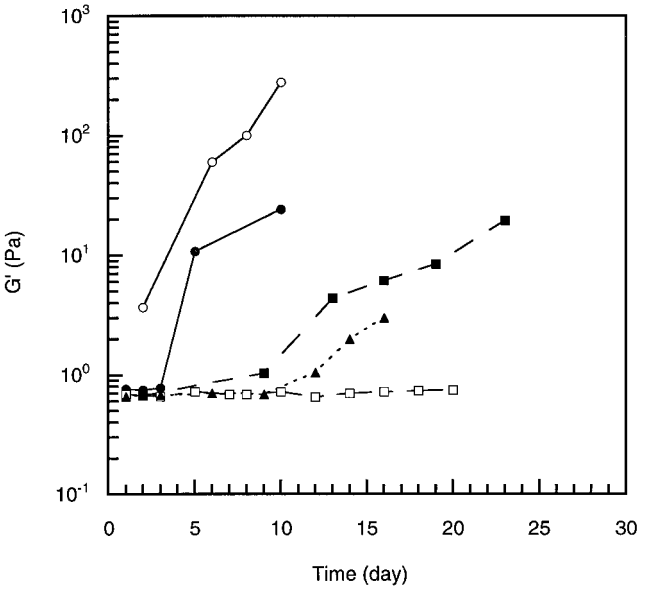

FIGURE 3 Effect of storage and the source of PrepA actin monomers on mechanical properties of actin filaments. The graph shows the time course of changes in G′ during storage of actin monomers. Mechanical properties were measured at a frequency of 0.1896 Hz in the Weissenberg rheometer. PrepA actin was purified from the 1989 acetone powder (●, 1 mg/ml; ○, 2 mg/ml) or from the 1992 acetone powder (■, ▲, and □, all 1 mg/ml) and stored at 4°C in a capped plastic tube for the indicated times.

the actin, G′ of filaments of PrepA actin is the same before and after gel filtration (Table 2), as reported previously for PrepB actin (Janmey et al., 1994).

Storage conditions

Actin can be stored without modifying its rheological properties either at 4°C (PrepA) or frozen (PrepB), but both methods require care. PrepB actin monomers were frozen at high concentration (5–10 mg/ml) in small volumes (<100 μl) and stored at −80°C. At the time of use, frozen samples were thawed rapidly at 37°C, clarified, and polymerized. Carefully frozen PrepB actin, stored for up to 1 year, had the same G′ as fresh actin. Slower freezing or thawing led variably to unpolymerizable actin or to actin that gelled in the low-ionic-strength G buffer. For example, in two of three experiments in which gel-filtered PrepA actin was frozen in 1-ml aliquots in liquid nitrogen, G′ was higher after freezing and thawing (Table 2).

PrepA actin monomers were stored at 4°C. Fresh from gel filtration, all samples of PrepA actin had the same rheological properties. Storage in buffer G1 at 4°C in a capped tube changed most PrepA actin samples such that after polymerization both G′ and G″ were higher than filaments of fresh actin (Fig. 3). The onset of these changes varied from 4 days with one acetone powder to more than 3 weeks in another (Fig. 3). In a single experiment, storage of PrepB actin monomers at 4°C in a capped tube followed by polymerization also resulted in higher values of G′. Storage in a capped tube did not degrade actin monomers (Fig. 4 *inset*) or change their critical concentration for polymerization for at least 1 week. However, some of the monomers aggregated, forming a gel filtration peak with a Stokes'

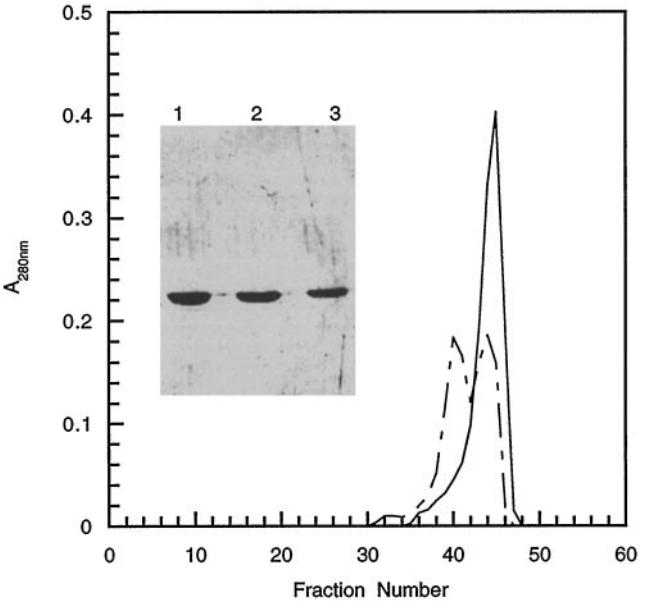

FIGURE 4 Gel filtration and gel electrophoresis of fresh actin and aged PrepA actin. Actin was gel filtered in buffer G1 on Sephacryl S-300 either fresh (——) or after storage in a capped plastic tube for 12 days (— - —). Protein was detected by absorption at 280 nm. (*Inset*) Ten percent polyacrylamide gel electrophoresis in SDS. Lane 1, fresh actin; lane 2, aged actin; lane 3, supernatant of polymerized aged actin.

radius of 4.6 nm that eluted ahead of the monomeric actin with a Stokes' radius of 3.9 nm (Fig. 4). Actin dialyzed against buffer G1 without change or against deionized water aggregated similarly. Co-polymerization of fresh actin with this aged actin increased its elastic modulus (Fig. 5).

These changes in actin monomers during storage can be partially reversed by ATP (Fig. 6) or avoided entirely by providing actin monomers with fresh buffer containing ATP and DTT. When stored at 4°C in continuous dialysis versus fresh buffer G1 changed daily, monomeric actin polymerizes to form filaments with the same mechanical properties as those assembled from fresh actin filaments. Under these conditions, neither G′ nor G″ changed more than 5% during storage for 4 weeks (Fig. 7). Actin purified from five acetone powders was stable under these conditions. On the

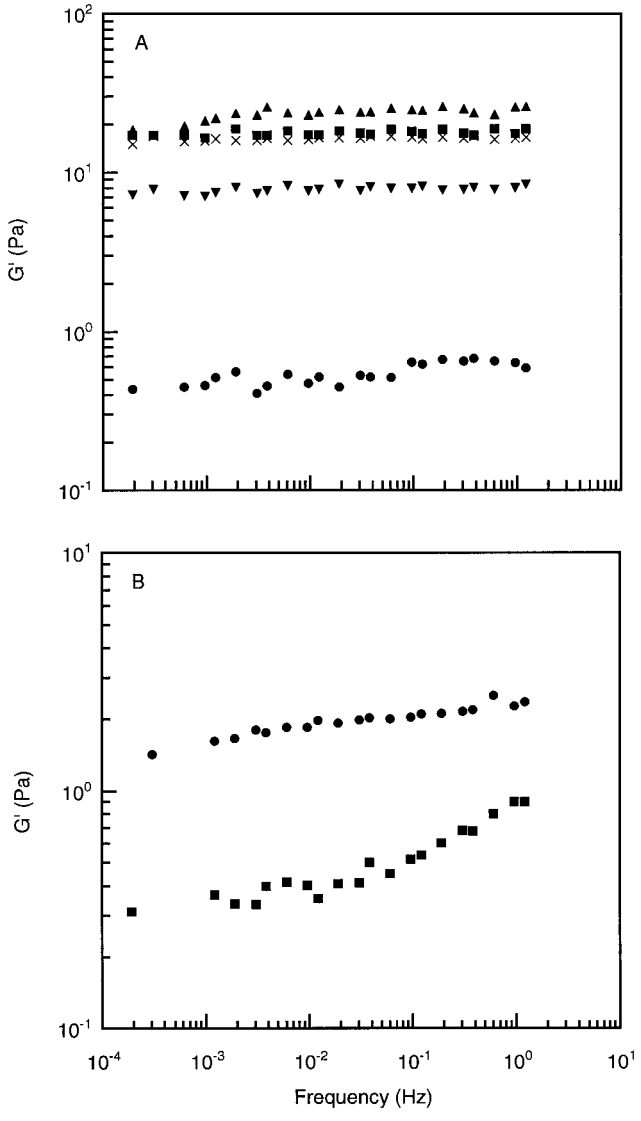

FIGURE 5 Aged actin alters the mechanical properties of fresh PrepA actin filaments. (*A*) Fresh actin was stored in a capped plastic tube and used within 2 days. The aged actin was stored in a capped plastic tube for 25 days. Mechanical properties were measured on 1 mg/ml mixtures of the two samples: 100% fresh actin (●), 70% fresh actin and 30% aged actin (▼), 50% fresh actin and 50% aged actin (×), 30% fresh actin and 70% aged actin (■), and 100% aged actin (▲). (*B*) Actin purified from the 1992 acetone powder and used fresh or stored in a capped plastic tube for 15 days. This aged actin was polymerized in 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM Tris (pH 8.0) added to buffer G1. Centrifugation at 100,000 × *g* for 2 h pelleted the actin filaments. The supernatant containing unpolymerized actin was used for this experiment. Mechanical properties were measured on actin filaments at 0.61 mg/ml. ●, G′ of a mixture of supernatant containing unpolymerized actin (40% of mass) and fresh actin (60% of mass); ■, G′ of fresh actin filaments alone.

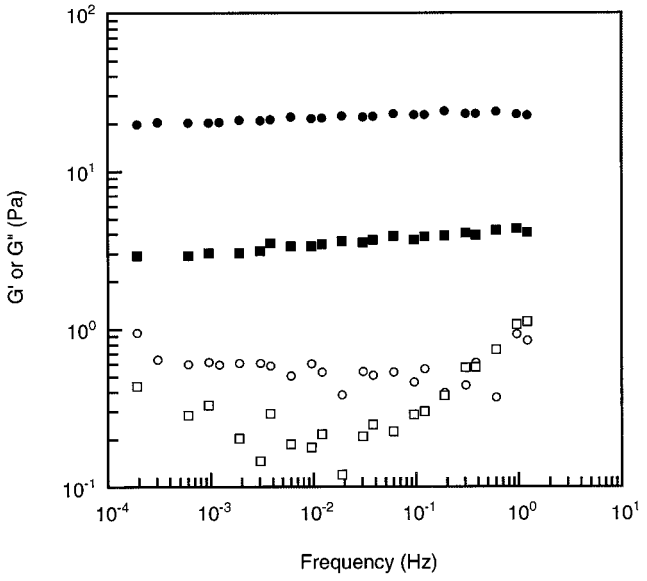

FIGURE 6 Partial reversal of changes during storage by ATP. PrepA actin from the 1989 acetone powder was stored at 4°C in a capped plastic tube. ● and ■, G′; ○ and □, G″. Actin was polymerized at 1 mg/ml after storage for 10 days (● and ○) or for 13 days, but 0.4 mM ATP was added 1 day before polymerization (■ and □).

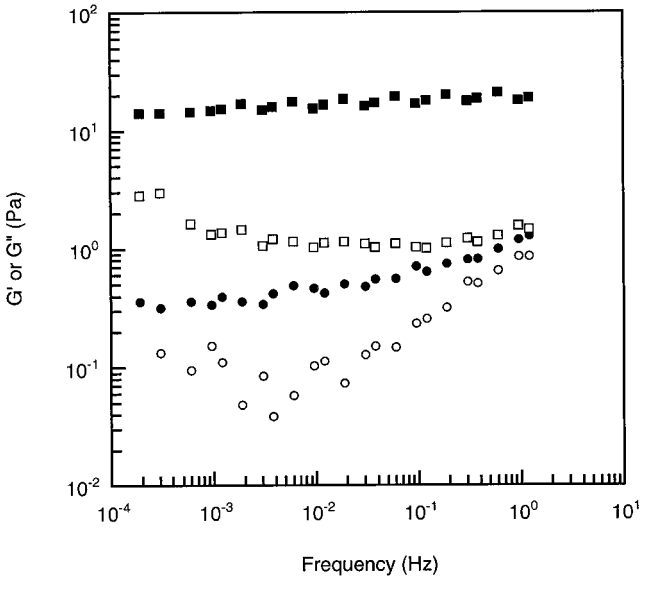

FIGURE 7 Effect of dialysis of actin monomers on the mechanical properties of actin filaments. PrepA actin monomers were stored in dialysis bags for 4 weeks. The dialysis buffer G1 was changed every day (● and ○) or never (■ and □). ● and ■, G′; ○ and □, G″.

other hand, after storage of actin monomers by dialysis against buffer G1 without changing or by dialysis against deionized water, the actin filaments had high values of G′ and G″ (Fig. 7), similar to actin stored in a capped tube.

DISCUSSION

Importance of the absolute values of the mechanical properties of actin filaments

A coherent description of the viscoelastic properties of actin filaments is valuable for a number of reasons. Most directly relevant to cell biology, it provides a baseline to consider the effects on cell mechanics of different classes of actin-binding proteins. Because cytoskeletal polymers are much stiffer and form gels at much lower volume fractions than common synthetic polymers, the well developed theories of rubber-like networks composed of flexible chains do not provide a conceptual framework to predict the material properties of the cytoskeleton. Reliable rheological studies of actin filament networks are required to evaluate the new theories for semiflexible polymers (MacKintosh et al., 1995; Isambert and Maggs, 1996; Satcher and Dewey, 1997).

One model for actin rheology (Satcher and Dewey, 1997) is based on a cubic lattice constructed of cross-linked actin filaments that bend under strain to generate a shear modulus of 10,000 Pa at 10 mg/ml. The shear modulus is proportional to the square of the polymer concentration, implying $G' = 100$ Pa at 1 mg/ml. The absence of cross-links between filaments must contribute to the lower values of G′ measured in the present work. In addition, the filaments assumed in the model may be stiffer or longer than the real filaments, so they form a better three-dimensional lattice.

Two other theories (MacKintosh et al., 1995; Isambert and Maggs, 1996) consider the origin of the elastic response of an actin filament solution to be the interplay between single filament bending elasticity and loss of entropy due to the reduction of Brownian motions of a filament under shear. These models, although not yet providing a quantitative prediction for the shear modulus of 1 mg/ml actin filaments, do predict that this quantity will depend very strongly on subtle differences in network structure. In particular, all models point out that the shear elasticity of an actin filament solution is a sensitive function of the bending elasticity of its constituent filaments, the presence of cross-links between filaments, and polymers long enough to become sterically intertwined.

Variables that affect the mechanical properties of purified actin filaments

The aim of the current collaboration was to resolve the long-standing differences in the measured mechanical properties of actin filaments reported in the literature. We considered a number of variables, including rheometers, polymerization buffers, and methods used to prepare and store acetone powders and the actin solutions. We conclude that a number of minor differences, when combined together, can result in substantial differences in the observed properties. Methods used to store actin monomers can produce major differences, but these artifacts can be avoided with proper care. Shear alignment can affect G′, but given the very low-amplitude strains (<2%) employed here and in previous studies, it cannot explain the historic differences.

Rheometers

The two rheometers most commonly employed in earlier work agreed within a factor of 3 when measuring identical samples. This difference was greater than the experimental error of repeated measurements with either instrument but does not account for the much larger difference seen when different preparations are assayed.

Polymerization conditions

Actin polymerized in buffers that yielded filaments containing predominantly either tightly bound Ca^{2+} or Mg^{2+} differed modestly in shear moduli. On average, G′ of Ca-actin filaments was 23% higher than Mg-actin filaments. Therefore, the influence of polymerization buffers on bound divalent cation does not account for the large differences reported previously. Polymerization is slower in the KMC7.4 buffer, so the filaments might be somewhat longer, although we did not observe a difference in filament length by light microscopy. Ca-actin filaments may be stiffer than Mg-actin filaments (Orlova et al., 1993, 1995), but this was not confirmed by Isambert et al. (1995) or Scharf and Newman (1995) or supported by our rheological data.

Actin purification

Preparation methods have a modest impact on the mechanical properties of actin filaments. Acetone powders prepared by different methods and stored at −20°C for 0–3 years yielded PrepA actin with identical mechanical properties. Gel filtration during PrepA removes minor contaminants that raise or lower the viscosity of the purified actin filaments (MacLean-Fletcher and Pollard, 1980; Casella et al., 1995). These minor contaminants may account for the higher G′ of fresh PrepB actin prepared without gel filtration, but the effects of the minor contaminants appear to cancel out for the most part.

The combined effects of the two- to threefold difference between rheometers, the 23% difference in the polymerization buffers, and a twofold difference in the actin preparations can account for a difference of five- to eightfold in G′ between the laboratories.

Storage

The viscoelasticity of actin filament networks is far more dependent on the details of sample storage than we origi-

nally appreciated. Storage of monomeric actin without frequent buffer changes can alter the mechanical properties of actin polymers by more than an order magnitude. These effects of storage alone do not explain previous discrepancies, as no previous work was done with actin that had been stored for periods more than a few days.

Fortunately, storage artifacts can be avoided; the mechanical properties of actin filaments are stable for weeks if the monomeric actin is stored by dialysis against buffer G containing ATP and DTT that is changed regularly. Alternatively, freezing can preserve the properties of the monomeric actin during storage. Care is required, because freezing and thawing monomeric actin before polymerization can alter the kinetics of the rise in G′ and the steady-state value of G′.

The nature of the change that occurs on aging is unknown. These differences in the actin are subtle, because all preparations appeared homogeneous by gel electrophoresis (Fig. 4) and polymerized with low critical concentrations. The filaments with different mechanical properties appeared similar by fluorescence microscopy after staining with rhodamine-phalloidin. Although the changes during storage may be physiologically unimportant and complicate experimental analysis, it is possible that defining the structural alteration that produces the rheological differences may reveal additional aspects of the complex and dynamic organization of actin networks.

Because the increase in G′ during storage is prevented by fresh buffer and reversed partially by addition of ATP, the change may be related to the previously reported difference in shear modulus between ATP and ADP actin. Monomeric actin in buffer G1 hydrolyzes ATP at a rate of 10^{-5} s^{-1} $monomer^{-1}$ at 21°C (Pollard and Weeds, 1984), so 50 μM actin will hydrolyze part of the 200 μM ATP in the buffer over a week even at 4°C. This rise in free ADP may contribute to the slow denaturation of the actin (Drewes and Faulstich, 1991) and the higher G′. The lower concentration of ATP in PrepA (200 μM) than prepB (500 μM) may make PrepA more susceptible to such age-dependent changes. In this model, denatured actin would remain competent for polymerization but would acquire a new contact site to which other filaments would adhere. Fluorescence micrographs show that rhodamine-phalloidin-labeled actin filaments in polymerizing buffer containing ADP instead of ATP have a tendency to cluster (Käs et al., 1996a). This finding suggests that parts of the unpolymerizable fraction of aged actin may function as transient filament cross-linkers. Steinmetz et al. (1997) have observed links between filaments of highly purified actin by electron microscopy and related them to actin dimers that form transiently in the presence of divalent cations.

The recent theoretical model for the viscoelasticity of entangled, semiflexible chains by MacKintosh et al. (1995) suggests another possibility. Due to the stiffness of actin filaments, not each contact point in the polymer mesh is also an entanglement point that resists deformation. Only filaments with a length exceeding the average length between two entanglement points contribute to the elastic response of the mesh. For realistic parameters of filament stiffness and concentration, filaments must be at least several microns long to contribute to the elasticity (MacKintosh et al., 1995; Isambert and Maggs, 1996; Maggs, 1997). These effects are especially important at the low volume fractions used in our studies (0.1%) where the formation of entangled networks is marginal. Filaments below a critical length, which could be several microns, would not contribute to the elastic modulus, whereas filaments above that length would contribute disproportionately to the modulus in a manner that increases with length. Kinks or defects in actin filaments resulting from aging might promote entanglements in addition to the more gentle topological constraints caused by the other filaments surrounding each strand as a tube-like constraint. Aging might promote contacts between the filaments either by nonspecific associations (as observed by gel filtration) or by formation of disulfide bridges between subunits in different filaments. Such entanglements would be similar to specific cross-links and would increase G′ significantly. Although fluorescence microscopy of rhodamine-phalloidin-stained actin filaments did not reveal gross differences in filament length or points of high curvature, subtle differences in length distribution may exist or may be prevented by the antioxidants and sugars present in the anti-bleach buffers necessary for microscopy.

Implications for future work

The important point for future studies is that networks of actin filaments can be prepared reproducibly that have G′ values of ~1 Pa for 1 mg/ml. This value is sensitive not only to the presence of actin-binding proteins but also to subtle and still undefined properties of actin itself. Careful storage of monomeric actin in fresh buffer with ATP and DTT makes the measurements highly reproducible and avoids the changes that produce larger values for the dynamic elastic modulus. Inclusion of a capping protein to control polymer length also improves reproducibility.

This collaborative investigation studied highly purified actin polymers. Theoretical inferences based on the kinetics of actin assembly borne out by experiments have shown that such actin preparations may contain extremely long filaments (Pollard, 1983; Oosawa, 1993; Käs et al., 1996b), and such filaments are subject to lateral alignments on the basis of steric and ionic interactions (Suzuki and Ito, 1991; Furukawa et al., 1993; Tang and Janmey, 1996). In addition, the average lengths of individual filaments change very slowly as a result of filament breakage and annealing or subunit exchange at filament ends. These phenomena contribute to the complexity of rheological measurements and might be influenced by the experimental conditions explored in this study. In repeated examinations, however, the elastic properties of actin filaments track monotonically with their average lengths regulated by actin filament sev-

ering and barbed end capping proteins, and the elastic moduli undergo large increases when cross-linking agents ligate short actin filaments (Janmey et al., 1990a; Maciver et al., 1991). Therefore, despite its rich subtleties, which deserve further research, the problem of actin rheology becomes more tractable as the factors that control the structure of actin networks become better defined.

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