

Dynamic Cross-linking by α -Actinin Determines the Mechanical Properties of Actin Filament Networks*

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We used smooth muscle α -actinin to evaluate the contribution of cross-linker dynamics to the mechanical properties of actin filament networks. Recombinant actin-binding domain (residues 2–269) binds actin filaments with a K_d of 1 μM at 25 °C, 20 times stronger than actin-binding domain produced by thermolysin digestion of native α -actinin (residues 25–257). Between 8 and 25 °C the rate constants for recombinant actin-binding domain to bind to (0.8–2.7 $\mu\text{M}^{-1} \text{s}^{-1}$) and dissociate from (0.2–2.4 s^{-1}) actin filaments depend on temperature. At 8 °C actin filaments cross-linked with α -actinin are stiff and nearly solid, whereas at 25 °C the mechanical properties approach those of actin filaments alone. In these experiments, high actin concentrations kept most of the α -actinin bound to actin and temperature varied a single parameter, cross-linker dynamics, because the mechanical properties of pure actin filaments (a viscoelastic gel) or biotinylated actin filaments cross-linked irreversibly by avidin (a stiff viscoelastic solid) depend little on temperature. These results show that the rate of exchange of dynamic cross-links between actin filaments is an important determinant of the mechanical properties of the networks.

Cytoplasm is viscoelastic, so that it can either deform or rebound in response to force (1, 2). Deformation is essential for cellular movement, whereas elastic responses allow cells to maintain their shapes. Scientists have appreciated this duality of cytoplasm for more than 100 years (see Ref. 3 for a review of early work). To explain these complicated properties, Frey-Wyssling (4) proposed that cytoplasm consists of a network of long thin filaments held together by dynamic cross-links that can resist rapid deformation but allow slow deformation in response to sustained force. He did not know the molecular nature of the filaments or cross-links, because his suggestion, originally made in the late 1930s, predated the discovery of actin in muscle (5) and nonmuscle cells (6). Later Pollard and Ito (7) identified actin filaments as major contributors to the cytoplasmic gel, and Sato *et al.* (8) found that the mechanical properties of actin filament networks cross-linked with *Acanthamoeba* α -actinin depend on the rate of deformation. At low rates of deformation, a network containing α -actinin is no

stiffer than the actin filaments alone, but at high rates of deformation, the network is far stiffer than actin alone. They postulated that amoeba α -actinin forms dynamic cross-links between actin filaments and suggested that the Frey-Wyssling model might explain the ability of cells to resist rapid deformation but change shape in response to a slowly imposed force.

This hypothesis raised the question of whether dynamic cross-linkers are a general feature of actin filament networks. Janmey *et al.* (9) reported that actin-binding protein (ABP-280)¹ from a human smooth muscle tumor is not a dynamic cross-linker. ABP/actin gels have a higher elastic modulus than gels of actin filaments alone at all frequencies tested, similar to biotin-labeled actin filaments cross-linked irreversibly by avidin (10). Much lower concentrations of ABP than amoeba α -actinin were needed to affect the modulus of actin gels. They argued that if cytoplasmic actin filaments were cross-linked by proteins like ABP-280, then rearrangement of cross-links could not account for the properties of cytoplasm. This work emphasized the need to explore the effects of cross-linkers with a range of affinities for actin. The affinity of macrophage and smooth muscle ABP-280 for actin is about 1 μM (11–13).

Wachsstock *et al.* (15) compared the mechanical properties of actin filaments cross-linked with biotin-avidin ($K_d = 10^{-15} \text{ M}$; Ref. 10), smooth muscle α -actinin ($K_d = 0.6 \mu\text{M}$; Ref. 14 and 15), or *Acanthamoeba* α -actinin ($K_d = 5 \mu\text{M}$; Ref. 16). Biotin-actin/avidin gels are a viscoelastic solid at all frequencies, and concentrations of avidin $>0.03 \mu\text{M}$ do not increase the stiffness. At 0.03 μM smooth muscle α -actinin increased the complex modulus 10-fold less than biotin/avidin. At higher concentrations, smooth muscle α -actinin makes bundles of actin filaments that behave like a viscoelastic fluid (14, 16). High concentrations of amoeba α -actinin are required to increase the stiffness, and the complex modulus depends dramatically on frequency.

The current work investigates actin filament cross-linking by α -actinin in more detail to determine the effect of cross-linker dynamics on the mechanical properties in a system with only one variable, the temperature. We used chicken smooth muscle α -actinin, because temperature affects its affinity for actin filaments (17, 18). Previous studies examined the effect of temperature of gels of actin and α -actinin (19–21), but this is the first to correlate rheological measurements and binding rate constants on the same preparations of proteins. α -Actinins consist of two identical polypeptides of 103 kDa with an N-terminal 30-kDa actin-binding domain, a tail with triple helical repeats, and two C-terminal EF hands (22). The rod-shaped tails associate to form an anti-parallel dimer with an actin filament-binding site on each end. The bivalent protein cross-links actin filament into three-dimensional gels or bundles depending upon the conditions (16).

¹ The abbreviations used are: ABP, actin-binding protein; ABD, actin-binding domain; rABD, recombinant ABD.

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We used the monovalent actin-binding domain (ABD) for our kinetic analysis to avoid complications arising from two binding sites. We assume that the properties of isolated ABD are the same as the heads of bivalent α -actinin molecules. We initiated these experiments with ABD produced from chicken smooth muscle α -actinin by proteolytic digestion (14) but found that this fragment differs in size and affinity for actin compared with full-length recombinant ABD, which also provides a convenient fluorescence change when it binds actin filaments (18).

We show that actin filament networks cross-linked by α -actinin differ fundamentally from biotin-actin/avidin gels. Biotin-actin/avidin gels are viscoelastic solid at all frequencies and temperatures tested. α -Actinin/actin gels are less stiff, and the stiffness varies with temperature, because cross-linker dynamics depend on the temperature.

MATERIALS AND METHODS

Protein Purification—Actin was prepared from rabbit skeletal muscle (23, 24) using Sephacryl S-300 instead of Sephadex G-150 for gel filtration. α -Actinin was purified from chicken smooth muscle (25). Both actin and α -actinin were stored by dialysis against daily changes of Buffer G (2 mM Tris, pH 8.0, at 25 °C, 0.2 mM ATP, 0.1 mM CaCl₂, 0.5 mM dithiothreitol, 0.3 mM NaN₃) and used within 5 days of purification. Lyophilized avidin (Pierce) was dissolved at 1 mg/ml in Buffer G. Actin was labeled with biotin as described by Wachsstock *et al.* (15) and Janmey *et al.* (9) by reacting polymerized actin with a 7-fold molar excess of iodoacetyl-*N*'-biotinhexenediamine (from a 40 mM stock in dimethylformamide). After purification by pelleting, depolymerization, and gel filtration, the extent of biotinylation was measured by the displacement of 2-[4'-hydroxyazobenzene] benzoic acid from avidin with a kit from Pierce. The actin was approximately 30% biotinylated. This actin was diluted to 2% biotinylated actin with unlabeled actin for the experiments.

Proteolytic Chicken Smooth Muscle α -Actinin ABD—The ABD of chicken smooth muscle α -actinin was cleaved from the tails by digestion with thermolysin coupled to agarose beads (Sigma) and purified according to Wachsstock *et al.* (15), a modification of the method of Pavalko and Burridge (26). The thermolysin beads were filtered out and the soluble pABD was recovered in the void volume of a 5-ml Econo-Q column (Bio-Rad) equilibrated with Buffer G. The column retains the 53-kDa rod domain. Dr. Wolfgang Fischer of The Salk Institute for Biological Studies determined the N-terminal sequence of this pABD by automated Edman degradation and determined its mass by mass spectrometry.

Recombinant Chicken Smooth Muscle α -Actinin ABD—Dr. D. R. Critchley (University of Leicester, UK) kindly provided an *NcoI-HincII* restriction enzyme fragment encoding the ABD of chicken smooth muscle α -actinin (residues 2–269, residue 1 = initiator met) cloned into expression vector pMW 172 (27). Recombinant ABD (rABD) was expressed in the BL21.de3 strain of *Escherichia coli* and purified by modifications of method Kuhlman *et al.* (18). Cells were sonicated in 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 200 mM NaCl, 1% Triton X-100, pH 8.0, at 25 °C and clarified. The supernatant was dialyzed against Buffer T (10 mM Tris, pH 8.0, at 25 °C, 10 mM NaCl, 2 mM EDTA, 0.05% β -mercaptoethanol) for 3 h. rABD was eluted with 60 mM NaCl from a 2 \times 16-cm DE-52 fast flow anion exchange column (Whatman International Ltd., Maidstone, UK) equilibrated with Buffer T. After precipitation with 60% ammonium sulfate, rABD was resuspended in 5 ml of Buffer G and chromatographed on a 1 \times 100-cm column of Sephadex G-75. The rABD peak was run on a MonoQ column (Bio-Rad) equilibrated with Buffer G and eluted with a linear gradient of 10–500 mM NaCl. The concentration of ABD was determined by absorbance using the extinction coefficient $A_{280} = 23.8 \mu\text{M}^{-1}\text{cm}^{-1}$ (27) or by the Bradford (28) method standardized with bovine serum albumin.

Stopped Flow Fluorescence Measurements—The rate constant (k_{obs}) for rABD binding to actin filaments was measured from the time course of the decrease in the intrinsic fluorescence after mixing equal volumes of the ABD and actin (18) in the stopped flow instrument described by Sinard and Pollard (29). Tryptophan fluorescence was excited at 289 nm with a short pass filter (Oriel, Stratford, CT), and emission was monitored using a 330-nm-long pass filter (Oriel, Stratford, CT). A thermocirculator controlled the temperature of the sample and the observation cell. Actin in Buffer G was polymerized for >2 h by adding one-tenth volume of 10 \times KME buffer (500 mM KCl, 10 mM MgCl₂, 10 mM EGTA,

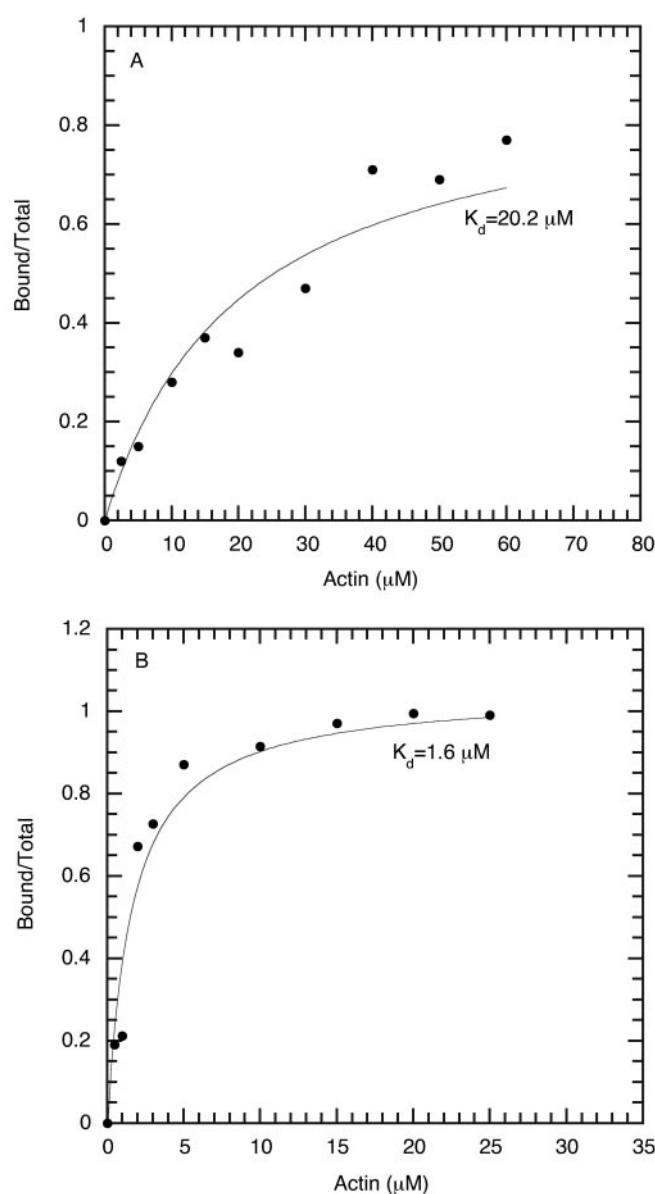


FIG. 1. Pelleting assay for pABD and rABD binding to skeletal muscle actin filaments. The conditions were 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM imidazole, pH 7.0, in Buffer G, 22 °C. A, 1 μM pABD pelleted with various concentrations of actin filaments. The fitted curve corresponds to a dissociation equilibrium constant (K_d) of 20.2 μM. B, 0.4 μM rABD pelleted with various concentrations of actin filaments. The K_d is 1.6 μM.

100 mM imidazole, pH 7.0). ABD was made up in the same buffer. Prior to loading the stopped flow syringes, proteins were diluted to desired concentrations with Buffer F (2 mM Tris, 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 0.2 mM ATP, 0.5 mM dithiothreitol, 0.3 mM NaN₃). After rapid mixing (dead time, 3 ms), we collected 500 data points at a sampling interval of 0.1 or 1 ms. Transients were fitted to single exponentials. Association rate constants (k_+) were determined from the dependence of k_{obs} on rABD concentration. rABD concentrations were at least 4-fold higher than actin.

Rapid Dilution Fluorescence Measurements—Rate constants for ABD dissociation from actin filaments were determined from the time course of the increase in intrinsic fluorescence after diluting the complex at least 20-fold with Buffer F. Measurements were made in a Photon Technology International Alphascan fluorescence spectrophotometer (Brunswick, NJ). The samples were diluted two ways: 1) syringe injection of 5 μl of complex into 3 ml of Buffer F in a 1 \times 1-cm cuvette rapidly stirred with a magnetic stirring bar or 2) using a hand-driven model SFA-12 Rapid Kinetics Stopped Flow Accessory (Hi-Tech Scientific Ltd., Salisbury, UK) with a dead time of about 20 ms. The temperature of the sample handling system was maintained with circulating water.

TABLE I
Rate and equilibrium constants for chicken smooth muscle α -actinin binding skeletal muscle actin filaments

The errors cited for k_- are standard deviations. SF, stopped flow; ND, not determined.

Temperature °C	k_+ $\mu\text{M}^{-1} \text{s}^{-1}$	k_- s^{-1}	K_d μM	Method	Reference
Recombinant actin-binding domain					
8	0.8	0.2 ± 0.04	0.3	SF/fluorescence	Current work
15	1.4	0.8 ± 0.04	0.6	SF/fluorescence	Current work
25	2.7	2.4 ± 0.1	0.9	SF/fluorescence	Current work
5	0.2	0.5	ND	SF/fluorescence	18
15	1.8	1.5	0.8	SF/fluorescence	18
25	4.1	9.7	2.4	SF/fluorescence	18
25			1.6	Pelleting	Current work
Room temperature			4.7	Pelleting	27
Proteolytic actin-binding domain					
22			20.2	Pelleting	Current work
22			19.5	Pelleting	14
22	3.0	57	19	SF/fluorescence polarization	15
Native α -actinin					
22			0.6	Pelleting	15
22			0.6	Pelleting	16
20	1.0	0.44	0.44	SF/NBD-actin fluorescence	13

Actin and ABD were copolymerized by adding one-tenth volume of concentrated polymerized buffer $10\times$ KME for at least 2 h. The time course was fitted with a single exponential.

Actin Filament Pelleting Experiments—Various concentrations of actin with a fixed concentration of ABD were polymerized for 1 h at room temperature in $1\times$ KME buffer and then centrifuged $200,000 \times g$ for 40 min at 25°C . The supernatants were concentrated by precipitation with chloroform/methanol, and the proteins were separated by polyacrylamide gel electrophoresis in SDS. After staining with Coomassie Blue, the gel was digitized using the program Adobe PhotoShop 3.0, and the integrated densities of the ABD bands were measured with the program NIH Image 1.6. These densities were converted to concentrations using ABD standards on the same gel, and the binding isotherm was determined by a nonlinear least squares fit of the data to a hyperbola.

Rheology—The rheological measurements were made with a parallel plate Rheometrics RFS II rheometer (Rheometrics Inc., NJ) in the small amplitude (strain, $\leq 2\%$) forced oscillation mode (30). Monomeric actin was mixed with one-tenth volume of $10\times$ KME polymerization buffer and immediately placed between the metal plates of the rheometer to polymerize at desired temperature. The plates were sealed with mineral oil (Sigma) to prevent sample dehydration. Measurements of G' and G'' were made every 30 s using time sweep mode to observe the gel formation. After G' and G'' reached a plateau, frequency sweep mode was used to measure the rheological parameters: the magnitude of complex modulus $|G^*|$, where $|G^*| = (G'^2 + G''^2)^{1/2}$; and the phase shift δ , where $\delta = \tan^{-1}(G''/G')$ (31). A solid has a phase shift of 0. A viscous liquid has phase shift of 1.6 radians. Relaxation experiments measured stress relaxation ($\tau(t)$) after a step input strain (γ_0) as a function of time. The relaxation modulus $G(t) = \tau(t)/\gamma_0$. Unlike a dynamic mechanical assay, a stress relaxation assay does not require deformation of the actin network after the initial strain.

RESULTS

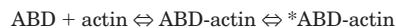
Comparison of Recombinant and Proteolytic Actin-binding Domain of Smooth Muscle α -Actinin—Both recombinant and proteolytic ABD preparations bind actin filaments in pelleting experiments (Fig. 1), but the affinities for actin filaments differ by an order of magnitude (Table I). At room temperature, the K_d values are $1.6 \mu\text{M}$ for rABD and $20 \mu\text{M}$ for pABD. This establishes that the wide range of affinities reported in the literature (Table I) are due to the methods of preparation and not differences in the assays.

Although both ABDs bind actin filaments, the intrinsic fluorescence of the complexes differs. Under a variety of conditions the intrinsic tryptophan fluorescence of mixtures of pABD and actin filaments is the same as the sum of the fluorescence of the separate proteins. On the other hand, we confirmed that mixtures of rABD and actin filaments have at least 5% lower intrinsic tryptophan fluorescence than the sum of the individual proteins (18). This fluorescence change makes rABD useful

for kinetic analysis of the binding reaction.

The two ABD preparations also differ in primary structure. Proteolytic ABD has a higher mobility on SDS-polyacrylamide gel electrophoresis (M_r 27 kDa) than the rABD (M_r 32 kDa). By Edman degradation the N-terminal sequence of pABD is LLD-PAXEKQQRKXFYA, identical to residues 25–40 (initiator M = 1) coded by the cDNA clone (32). By mass spectroscopy, the monoisotopic mass of pABD is 26,532, so its C terminus is Glu²⁵⁷. Recombinant ABD consists of residues 2–269 (27), including Trp²⁰, which may contribute to the fluorescence change associated with actin filament binding.

Kinetics of α -Actinin ABD Binding to Actin Filaments—We followed the binding of rABD to actin filaments under pseudo-first order conditions with a molar excess of rABD over polymerized actin subunits. Following rapid mixing in the stopped flow instrument, rABD binds to actin filaments accompanied by a quench in tryptophan fluorescence that follows an exponential time course (Fig. 2A), as first reported by Kuhlman *et al.* (18). The pseudo-first order rate constant of the fluorescence change depends linearly on rABD concentration up to a rate of at least 80 s^{-1} (Fig. 2B). A simple model for the reaction is a bimolecular binding event followed by a first order change in fluorescence.



REACTION 1

The absence of curvature in the plots of k_{obs} versus rABD shows that the fluorescence change is much faster than binding under our conditions ($>80 \text{ s}^{-1}$). The slopes of these plots at three temperatures give the apparent second order association rate constants (k_+) (Table I).

We measured the dissociation of rABD from actin filaments after dilution of the complex. Dissociation is accompanied by an increase in tryptophan fluorescence (Fig. 3). These data are noisy due to the very low concentrations of protein after dilution but could be fit with a single exponential. The dissociation rate constants are more temperature-dependent than the association rate constants (Table I). The dissociation equilibrium constants (K_d) are calculated from the ratio of the rate constants (Table I).

Mechanical Properties—Actin filament networks are viscoelastic materials with mechanical properties that are strongly influenced by cross-linking proteins. This study addresses the hypothesis (8, 16) that the rate of cross-linker

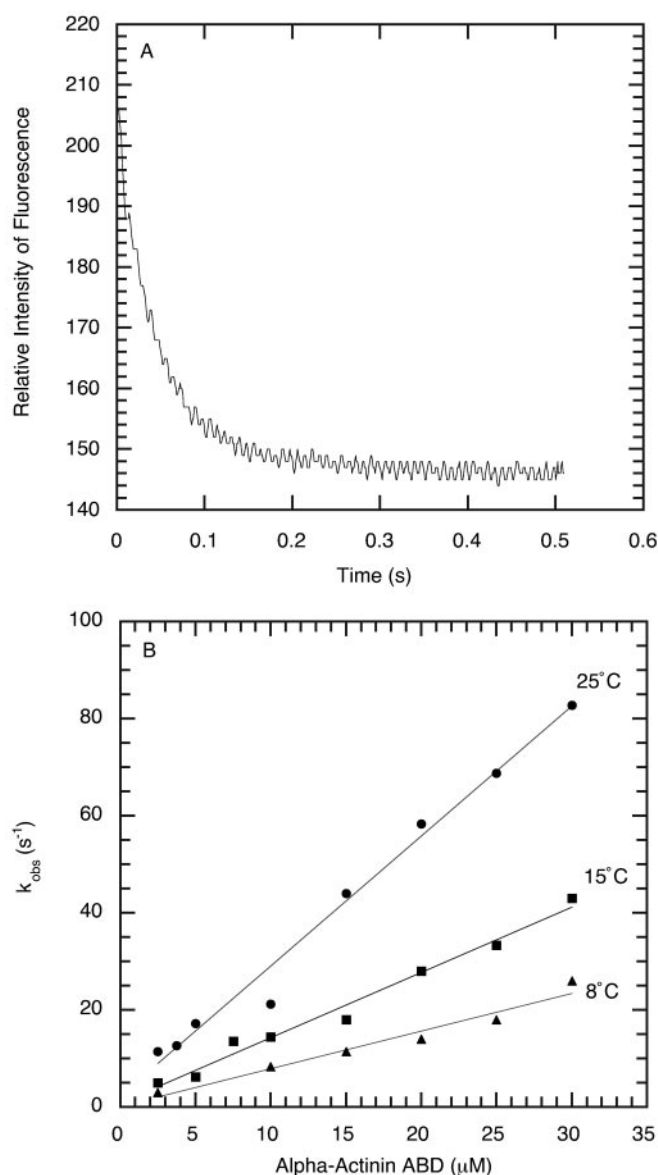


FIG. 2. Kinetics of the association of rABD with skeletal muscle actin filaments. The conditions were 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM imidazole, pH 7.0, in Buffer G. *A*, time course of the change in tryptophan fluorescence during the reaction of 10 μM actin-binding domain with 2 μM actin at 25 °C. The data fit a single exponential, giving a pseudo-first order rate constant of 25 s⁻¹. *B*, dependence of the pseudo-first order rate constant for ABD binding to actin filaments on ABD concentration and temperature. (The points are the average of at least four runs.) Slopes are the association rate constants summarized in Table I.

dissociation is a major determinant of the mechanical properties of actin gels. The particular test was to use temperature to vary the dissociation rate constant of the well characterized cross-linker smooth muscle α-actinin and to measure how this single parameter influences the mechanical properties of a cross-linked actin gel, under conditions where network is isotropic and the actin concentration is high enough that most α-actinin is bound at all temperatures.

First we determined how temperature influences the mechanical properties of actin filaments alone and irreversibly cross-linked actin filaments. We used avidin to cross-link actin filaments containing a few subunits carrying biotin, a high affinity ligand for tetravalent avidin (9–14). The earlier work showed that such tightly cross-linked actin filaments form a stiff, solid gel independent of the rate of deformation. The K_d

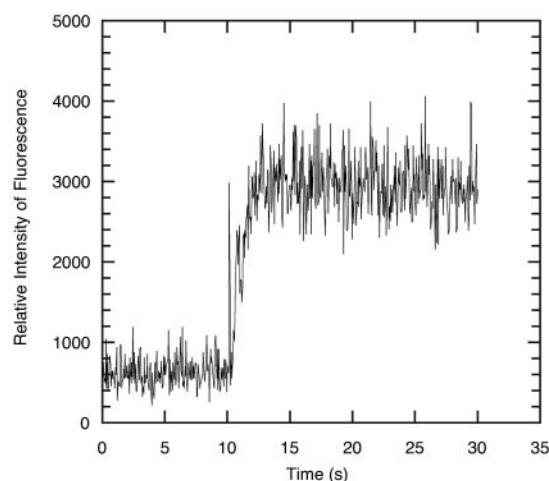


FIG. 3. Time course of dissociation of rABD from skeletal muscle actin filaments. The conditions were 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM imidazole, pH 7.0, in Buffer G. Shown is the time course of increase in tryptophan fluorescence after diluting the sample from 10 μM α-actinin and 2 μM actin to 0.03 μM α-actinin and 0.006 μM actin at 25 °C. The data were fitted to a single exponential with a rate constant of 2.5 s⁻¹. The dissociation rate constants in Table I are the average of at least four runs.

for avidin and biotin is 10⁻¹⁵ M (10). Assuming $k_+ = 10^8$ M⁻¹ s⁻¹, k_- is about 10⁻⁷ s⁻¹, a negligible rate in the laboratory or cellular context.

Over a wide range of deformation frequencies, networks of biotinylated-actin filaments (Fig. 4) have the same mechanical properties as filaments of unmodified actin (Fig. 5). The complex modulus ($|G^*|$) measures stiffness, and phase shift (δ) measures whether the material behaves like a solid ($\delta = 0$), a liquid ($\delta = 1.6$ radians), or in between like many viscoelastic materials. These mechanical properties of actin and biotin-actin filaments vary by less than 10% with temperatures over the range of 8–40 °C, confirming the observations of Sato *et al.* (8) on actin.

Biotin-actin filaments cross-linked irreversibly by avidin are very stiff (Fig. 4A) with a phase shift close to zero, like a solid (Fig. 4B). The mechanical properties of these irreversibly cross-linked actin filament gels are independent of temperature in the range of 8–40 °C (Fig. 4, A and B). Thus the mechanical properties of the filaments themselves are not sensitive to temperature in this range.

In contrast to either filaments of pure actin or irreversibly cross-linked actin filaments, the mechanical properties of actin filament networks cross-linked by chicken smooth muscle α-actinin depend strongly on temperature (Figs. 5 and 6A). At 8 °C the networks are stiff and nearly solid. At 25 °C the networks approach the properties of actin alone. The mechanical properties of actin filaments cross-linked with amoeba α-actinin depend on temperature in a similar way (8). A relaxation experiment (Fig. 6B) confirmed these properties. $G(t)$ of actin filament networks relaxes much more quickly than networks of actin filaments cross-linked by α-actinin. At long times (10⁴ s) the plateau modulus of actin filaments alone is independent of temperature and much lower than actin filaments cross-linked by α-actinin. The plateau modulus of cross-linked filaments depends on temperature. The difference is particularly striking at 8 °C, where G is higher by a factor of 100. Thus α-actinin cross-links account for most of the elastic modulus, and the contribution of entangled filaments is negligible.

DISCUSSION

Comparison of Proteolytic and Recombinant ABD—This and previous work (14, 18) employed the isolated actin-binding

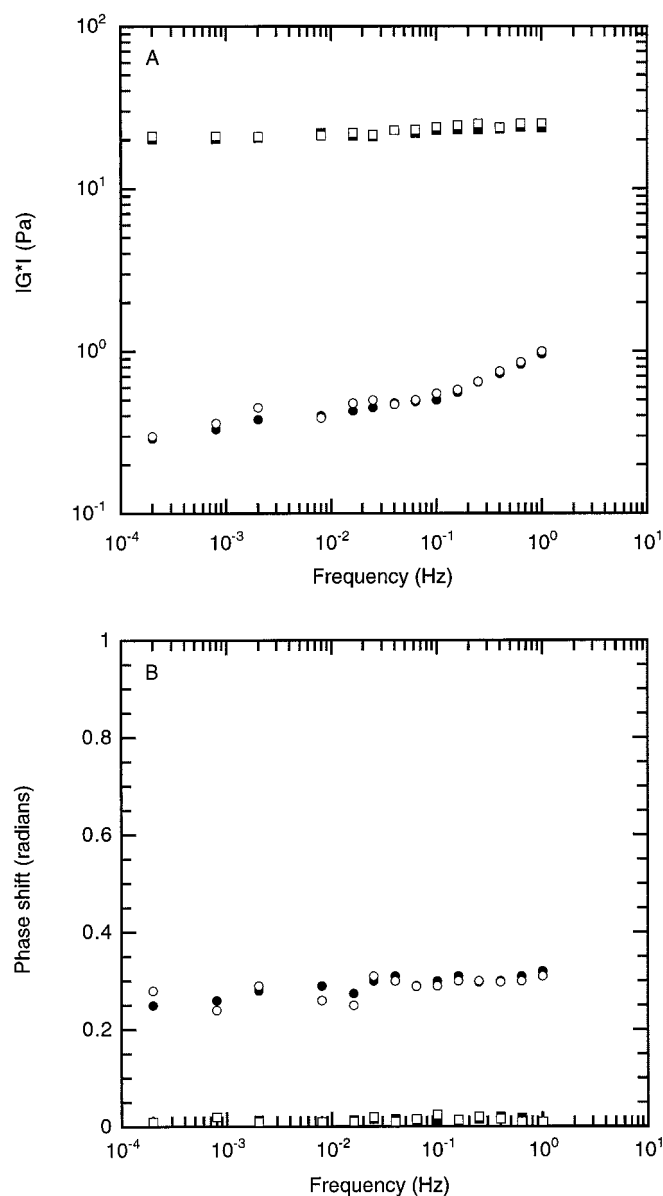


FIG. 4. The dependence of the mechanical properties of biotin-actin filaments and biotin-actin filaments cross-linked with avidin on temperature and the frequency of deformation. The conditions were 15 μM 2% biotinylated actin \pm 0.03 μM avidin polymerized in the rheometer in Buffer F. ●, 15 μM 2% biotinylated actin at 25 °C; ○, 15 μM 2% biotinylated actin at 8 °C; ■, 15 μM 2% biotinylated actin with 0.03 μM avidin at 25 °C; □, 15 μM 2% biotinylated actin with 0.03 μM avidin at 8 °C. A, complex modulus $|G^*|$. B, phase shift (δ).

domain of chicken smooth muscle α -actinin rather than the bivalent native molecule to evaluate the rates of actin filament binding and dissociation. In previous work on pABD of smooth muscle α -actinin, it was assumed that thermolysin simply cleaves the ABD from the α -helical tail (14, 26), because Baron *et al.* (32) found that isolated pABD did not yield any product upon Edman degradation, as expected for a fragment with an intact, blocked N terminus (33). However, Davison *et al.* (34) presented evidence that thermolysin actually cleaves between Leu²⁴ and Leu²⁵. We confirm this observation. By mass spectroscopy we show that thermolysin also cleaves after Glu²⁵⁷, suggesting that this is the end of the compactly folded head, rather than Phe²⁴⁶, which begins the spectrin-like repeats of the tail or Glu²⁶⁹, which seems to have been chosen as the end of rABD simply because of a convenient restriction site (27). The last 12 residues of pABD were not included in the crystal-

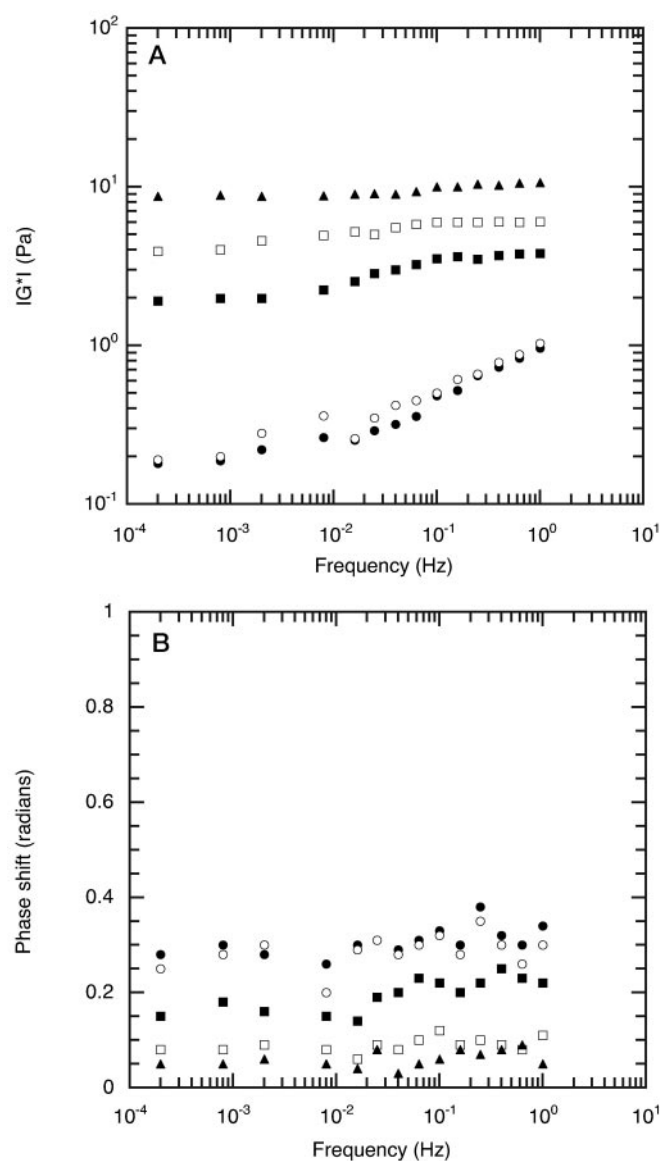


FIG. 5. The dependence of the mechanical properties of actin filaments cross-linked with smooth muscle α -actinin on temperature and the frequency of deformation. The conditions were 15 μM actin \pm 0.03 μM α -actinin polymerized in the rheometer in Buffer F. ●, 15 μM actin at 25 °C; ○, 15 μM actin at 8 °C; ■, 15 μM actin with 0.03 μM α -actinin at 25 °C; □, 15 μM actin with 0.03 μM α -actinin at 15 °C; ▲, 15 μM actin with 0.03 μM α -actinin at 8 °C. A, complex modulus $|G^*|$. B, phase shift (δ).

lized fimbrin ABD, but presumably link the α -actinin ABD to its tail.

Although an α -actinin mutant lacking amino acids 2–19 correctly localized to actin filaments and adhesion plaques in live cells (35), we find that truncated pABD produced by thermolysin digestion has a much lower affinity for actin filaments than rABD. Both equilibrium pelleting experiments and the ratio of the dissociation and association rate constants gave a K_d of 19–20 μM (14; present work) for pABD. By the same criteria rABD binds much tighter, with a K_d of 0.9–1.7 μM at 25 °C (Ref. 18 and present work). The main difference is the dissociation rate constant, which is 20 times higher for the proteolytic fragment (Table I).

The peptide missing from the N terminus of pABD is disordered in the atomic structure of the actin-binding domain-1 of human fimbrin (36), a homolog of α -actinin. The 18 disordered residues in the crystals of recombinant fimbrin ABD corre-

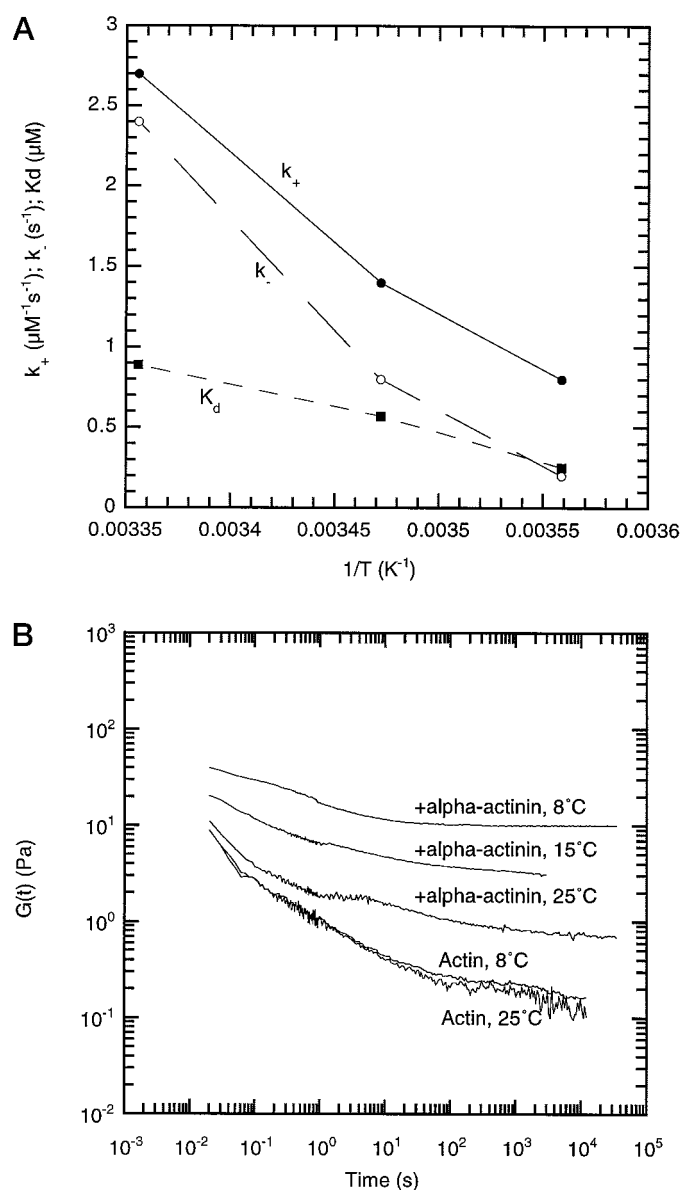


FIG. 6. *A*, dependence of dynamic rate constants and mechanical properties from Fig. 5 on temperature. *B*, relaxation experiment. The conditions were 15 μM actin ± 0.03 μM α-actinin polymerized in the rheometer in Buffer F at 25, 15, or 8 °C. At time 0, the samples were subjected to a step input strain of 1%, and *G* was followed as a function of time.

spond to residues 12–29 of α-actinin. The remainder of the crystallized ABD forms a compact α-helical structure consisting of two identically folded subdomains that differ in sequence. Thus the N-terminal peptide missing from pABD is not required for folding of the rest of the ABD.

Residues 1–24, present in rABD but not pABD, may account for the higher affinity of rABD for actin filaments, either directly by contacting actin or indirectly by stabilizing other actin-binding residues. The location of the actin-binding site is still an open question. Three parts of the ABD sequence were implicated as possible actin-binding sites by peptide binding or mutant analysis (37, 38), but each of these sequences is largely buried in the three-dimensional structure (36). The N-terminal peptide, on the other hand, must be on the surface of ABD. New data are required to define the actin-binding site more precisely.

The N-terminal residues missing from pABD may account directly for the lack of an effect of actin binding on its intrinsic

tryptophan fluorescence. Most likely, the fluorescence signal arises from a change in the environment of Trp²⁰ when rABD binds actin. One hypothesis is that upon binding actin, the disordered N terminus folds or undergoes a conformational change that exposes Trp²⁰ to solvent. Alternatively, residues 1–24 or 258–269 may be required to change the environment of tryptophans somewhere else in ABD and/or in actin. Experimental substitution of Trp²⁰ will help to distinguish these hypotheses.

Temperature Dependence of α-Actinin Binding to Actin Filaments—Both the association and dissociation rate constants for ABD-binding actin filaments depend on temperature (Table I). Our dissociation rate constants are smaller than those of Kuhlman *et al.* (18) using exactly the same proteins. Their values were based on extrapolation to zero rABD concentration of plots of *k*_{obs} versus rABD concentration in association experiments, a procedure with some error. In a confirmatory experiment they observed a rate constant of 15 s⁻¹ in a 1:10 dilution of 5 μM complex but did not investigate the dissociation reaction in a much detail as we have. As expected for a larger molecule, Goldman and Isenberg (13) found that intact smooth muscle α-actinin binds and dissociates somewhat more slowly at 20 °C than ABD (Table I).

The greater temperature dependence of the dissociation rate constant gives ABD a higher affinity for actin filaments at lower temperature, as observed since early studies of α-actinin (17). Kuhlman *et al.* (18) pointed out that the high activation energy for association (the activation enthalpy for association is about 11 kcal/mol; present work) suggests that binding involves a conformational change in the initial collision complex (perhaps in the N terminus of ABD) and that the association rate constant may not be limited by diffusion. Given its temperature dependence, dissociation may also involve a rate-limiting conformational change.

Cross-linker Dynamics Determine the Mechanical Properties of Actin Filament Gels—We assume that the two heads of α-actinin bind actin filaments with the same rate constants as rABD, with a small correction for slower diffusion of the larger native protein (14), but several factors complicate the situation. First, actin filaments are immobile on the time scale of the association reaction (39). Second, at the concentrations employed most univalent reactions of α-actinin with an actin filament will not position the second head appropriately to interact with a neighboring (immobile) actin filament. Third, prolonged dissociation of an α-actinin bound between two immobile actin filaments will be slower than two independent heads, because rebinding of a single dissociated head will be favored by being anchored to the neighboring filament. This may not complicate interpretation of mechanical properties, because dissociation of a single head breaks cross-links between filaments. Finally, force applied to a cross-link is likely to increase the rate of dissociation.

The current experiments using temperature provide the most direct support for the hypothesis that cross-linker dynamics, particularly dissociation rates, are an important determinant for the mechanical properties of actin filament gels. Temperature has little effect on the mechanical properties of actin filaments, whether free or cross-linked irreversibly. The modest effect of temperature on the affinity of α-actinin for actin allowed us to keep >94% of α-actinin bound. This leaves the dissociation rate constant and to a lesser extent the association rate constant as the main parameters that change with temperature and the most likely variable(s) to account for the dependence of the elastic modulus on temperature. When the cross-linker dissociation rate constant is low, the network is a stiff solid. As the dissociation rate constant of the cross-linker

increases with temperature, the stiffness and phase shift of the cross-linked networks of filaments approach those of actin alone. The earlier results of Wachsstock *et al.* (15, 16) also support the dynamic cross-linker hypothesis. However, their use of three different cross-linking proteins to provide a range of dissociation rate constants introduced more variables than the current work.

Our interpretation differs from Tempel *et al.* (21), who reported an extensive rheological analysis of actin filaments cross-linked by α -actinin as a function of temperature. They also found that these gels are stiffer at low temperature. Tempel *et al.* assumed that temperature varied the extent of association of α -actinin with the actin filaments but did not measure temperature dependence of the affinity directly. Instead, they derived the fraction of cross-links relative to a critical value from their rheological data, using a percolation theory that does not take into account the dynamics of the cross-links. If our equilibrium constants apply, >90% of the α -actinin was bound over the range of temperatures and actin concentrations that they examined. We suggest that the temperature dependence of cross-link dynamics may contribute to the effect of temperature on the elastic modulus in their experiments.

Because the effect of temperature is more pronounced in the relaxation experiments than in the dynamic experiments, the oscillating strain in the dynamic experiment may increase the rate of cross-linker dissociation. Because $G(t)$ measurements in the relaxation experiment do not strain the actin filament network during the assay, the values of the rate constants measured under conditions of zero strain should describe the equilibrium dynamics in the $G(t)$ experiments.

α -Actinin appears to differ from ABP-280/filamin (9), which is reported to form stiff networks at all frequencies despite the fact that it has association and dissociation rate constants and an equilibrium constant similar to those of α -actinin (13). The geometry of cross-linking may also differ in a way that impacts the mechanical properties (40). However, the observed difference may simply be due to the actin. The actin used in the filamin experiments had a much higher G' than the actin in the current study. A direct comparison of these cross-linking proteins with the same actin preparation is required to resolve these differences.

Implications for the Cell—The physical structure of the cytoplasm must adapt as a moving cell changes shape. There is little doubt that the actin cytoskeleton uses both filament assembly and disassembly as well as filament severing (and possibly annealing) to reconfigure. Because actin filaments themselves are quite stable kinetically (41), actin filament dynamics in the cell depend upon numerous actin-binding proteins that can sever, cap, nucleate, and cross-link the filaments.

Our current work provides support for the old idea that some of the adaptation of the actin cytoskeleton to change of shape is a passive response to force, allowed by the rapid exchange of some cross-links between the filaments. Our experiments confirm that the stiffness and phase shift of isotropic actin filament networks vary with the rate of dissociation of cross-links between the filaments. When force is applied slowly, dynamic cross-links allow the network to change shape. When force is applied suddenly, dynamic cross-links resist deformation and

store part of the energy for elastic recoil. Such dynamic cross-links may contribute to the ability of the stiff actin network in the cell cortex to change shape slowly during movements such as cytokinesis. The known effect of Ca^{2+} on the affinity of some cytoplasmic α -actinins for actin (42) may contribute to regulating the dynamics of these cross-links.

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REFERENCES

1. Bray, D., Heath, J., and Moss, D. (1986) *J. Cell Sci. (Suppl.)* **4**, 71–88
2. Elson, E. L. (1988) *Annu. Rev. Biophys. Biophys. Chem.* **17**, 397–430
3. Pollard, T. D. (1976) *J. Supramol. Struct.* **5**, 317–334
4. Frey-Wyssling, A. (1948) *Submicroscopic Morphology of Protoplasm and Its Derivatives*, Elsevier, New York
5. Straub, Y., and Feuer, G. (1950) *Biochim. Biophys. Acta* **4**, 455–470
6. Hatano, S., and Oosawa, F. (1966) *Biochim. Biophys. Acta* **127**, 488–500
7. Pollard, T. D., and Ito, S. (1970) *J. Cell Biol.* **46**, 267–289
8. Sato, M., Schwarz, W. H., and Pollard, T. D. (1987) *Nature* **325**, 828–830
9. Janmey, P. A., Hvidt, S., Lamb, J., and Stossel, T. P. (1990) *Nature* **345**, 89–92
10. Green, N. M. (1990) *Methods Enzymol.* **184**, 51–67
11. Hartwig, J. H., and Stossel, T. P. (1981) *J. Mol. Biol.* **145**, 563–581
12. Gorlin, J. B., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J., and Hartwig, J. H. (1990) *J. Cell Biol.* **111**, 1089–1105
13. Goldmann, W. H., and Isenberg, G. (1993) *FEBS Lett.* **336**, 408–410
14. Meyer, R. K., and Aebi, U. (1990) *J. Cell Biol.* **110**, 2013–2024
15. Wachsstock, D. H., Schwarz, W. H., and Pollard, T. D. (1994) *Biophys. J.* **66**, 801–809
16. Wachsstock, D. H., Schwarz, W. H., and Pollard, T. D. (1993) *Biophys. J.* **65**, 205–214
17. Holmes, G. R., Goll, D. E., and Suzuki, A. (1971) *Biochim. Biophys. Acta* **253**, 240–253
18. Kuhlman, P. A., Ellis, J., Critchley, D. R., and Bagshaw, C. R. (1994) *FEBS Lett.* **339**, 297–301
19. Grazi, E., Cuneo, P., Magri, E., Schwienebacher, C., and Trombetta, G. (1993) *Biochemistry* **32**, 8896–8901
20. Grazi, E., Trombetta, G., Magri, E., Cuneo, P., and Schwienebacher, C. (1994) *Biochem. J.* **298**, 129–133
21. Tempel, M., Isenberg, G., and Sackmann, E. (1996) *Physiol. Rev.* **54**, 1802–1810
22. Critchley, D. R. (1993) *α -Actinins: Guidebook to the Cytoskeletal and Motor Proteins* (Kreis, T., and Vale, R., eds) pp. 22–23, Oxford University Press, Oxford
23. Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
24. MacLean-Fletcher, S. D., and Pollard, T. D. (1980) *J. Cell Biol.* **85**, 414–428
25. Craig, S. W., Lancashire, C. L., and Cooper, J. A. (1982) *Methods Enzymol.* **85**, 316–321
26. Pavalko, F. M., and Burridge, K. (1991) *J. Cell Biol.* **114**, 481–491
27. Way, M., Pope, B., and Weeds, A. G. (1992) *J. Cell Biol.* **119**, 835–842
28. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
29. Sinard, J. H., and Pollard, T. D. (1990) *J. Biol. Chem.* **265**, 3654–3660
30. Sato, M., Leimbach, G., Schwarz, W. H., and Pollard, T. D. (1985) *J. Biol. Chem.* **260**, 8585–8592
31. Ferry, J. D. (1980) *Viscoelastic Properties of Polymers*, John Wiley & Sons, New York
32. Baron, M. D., Davison, M. D., Jones, P., and Critchley, D. R. (1987) *J. Biol. Chem.* **262**, 17623–17629
33. Singh, I., Goll, D. E., Robson, R. M., and Stromer, M. H. (1977) *Biochim. Biophys. Acta* **491**, 29–45
34. Davison, M. D., Baron, M. D., Wootton, J. C., and Critchley, D. R. (1989) *Int. J. Biol. Macromol.* **11**, 81–90
35. Hemmings, L., Kuhlman, P. A., and Critchley, D. R. (1992) *J. Cell Biol.* **116**, 1369–1380
36. Goldsmith, S. C., Pokala, N., Shen, W., Fedorov, A. A., Matsudaira, P., and Almo, S. C. (1997) *Nat. Struct. Biol.* **4**, 708–712
37. Bresnick, A. R., Janmey, P. A., and Condeelis, J. (1991) *J. Biol. Chem.* **266**, 12989–12993
38. Corrado, K., Mills, P. L., and Chamberlain, J. S. (1994) *FEBS Lett.* **344**, 255–260
39. Tait, J. F., and Frieden, C. (1982) *Biochemistry* **21**, 3666–3674
40. Janssen, K. P., Eichinger, L., Janmey, P. A., Noegel, A. A., Schliwa, M., Witke, W., and Schleicher, M. (1996) *Arch. Biochem. Biophys.* **325**, 183–189
41. Pollard, T. D. (1986) *J. Cell Biol.* **103**, 2747–2754
42. Bennett, J. P., Zaner, K. S., and Stossel, T. P. (1984) *Biochemistry* **23**, 5081–5086