Cross-Linker Dynamics Determine the Mechanical Properties of Actin Gels

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ABSTRACT To evaluate the contributions of cross-linker dynamics and polymer deformation to the frequency-dependent stiffness of actin filament gels, we compared the rheological properties of actin gels with three types of cross-linkers: a weak one, *Acanthamoeba* α-actinin (dissociation rate constant 5.2 s^{-1}, association rate constant 1.1 × 10^6 M^{-1} s^{-1}); a strong one, chicken smooth muscle α-actinin (dissociation rate constant 0.66 s^{-1}, association rate constant 1.20 × 10^6 M^{-1} s^{-1}); and an extremely strong one, biotin/avidin (dissociation rate constant approximately zero). The biotin/avidin cross-linked gel, whose behavior is determined by polymer bending alone, behaves like a solid and shows no frequency dependence. The amoeba α-actinin cross-linked gel behaves like a viscoelastic fluid, and the frequency dependence of the stiffness can be explained by a mathematical model for dynamically cross-linked gels. The stiffness of the chicken α-actinin cross-linked gel is independent of frequency, and has viscoelastic properties intermediate between the two. The two α-actinins have similar association rate constants for binding to actin filaments, consistent with a diffusion-limited reaction. Rigid cross-links make the gel stiff, but make it elastic without the ability to deform permanently. Dynamically cross-linked actin filaments should allow the cell to react passively to various outside forces without any sort of signaling. Slower, signal-mediated pathways, such as severing filaments or changing the affinity of cross-linkers, could alter the nature of these passive reactions.

INTRODUCTION

The cytoplasm is a viscoelastic material; it can either deform or rebound in response to an imposed stress (Bray and White, 1988; Elson, 1988). Control of this response is an important part of maintaining cell shape and allowing cell motility. Actin filaments and the proteins that cross-link them and control their size make a major contribution of these mechanical properties of cytoplasm. We have previously reported (Sato et al., 1987) that the presence of the cross-linking protein α-actinin from *Acanthamoeba castellanii* affects the physical properties of actin filament gels in a way that may help explain the properties of cytoplasm. At low rates of deformation, the gels containing α-actinin are no stiffer than gels of actin filaments alone, but at high rates of deformation the complex modulus, the measure of resistance to deformation, is far greater in the presence of α-actinin. If this is true in cytoplasm, it would allow the cell to resist deformation if the deformation were rapid, but change shape in response to a slowly imposed force, as has been observed (Bray et al., 1986; Rappaport, 1967).

Janmey et al. (1990) have reported that another actin cross-linking protein, actin-binding protein (ABP-280) from human uterine leiomyoma, a smooth muscle tumor, does not have this effect. ABP/actin gels have a higher modulus than gels of actin filaments alone at all frequencies. Much lower concentrations of ABP than α-actinin were needed to affect the modulus of actin gels, presumably because the ABP binds to actin more tightly. The dissociation equilibrium constant of ABP-280 from leiomyoma for actin filaments is unknown, but that of ABP-280 from macrophages is 1 μM (Gorlin et al., 1990), while that of amoeba α-actinin is 5 μM (Wachsstock et al., 1993). Janmey et al. compared the physical properties of ABP-280/actin gels to those of actin gels labeled with biotin and cross-linked by avidin. Biotin binds avidin with a dissociation equilibrium constant of 10^{-15} M, near that of a covalent bond (Green, 1990). The biotin-actin/avidin gel, therefore, is a gel with permanent cross-links, and its physical properties reflect those of the actin filaments themselves. The stiffness of the gel is due to bending or breaking of the filaments, not to sliding of one filaments past another. Janmey et al. showed that the physical properties of an ABP-280/actin gel at a particular concentration of cross-linker and rate of deformation were similar to those of a biotin-actin/avidin gel. They argued that rearrangement of actin filament cross-linking proteins does not account for the physical properties of cytoplasm. However, they did not explore a range of cross-linker concentrations or rates of deformation.

We have previously reported (Wachsstock et al., 1993) that the structure of cross-linked actin filament gels depends strongly on the affinity of the cross-linker for the actin. A weak cross-linker such as *Acanthamoeba* α-actinin will form bundles of actin filaments, which are stiff and have a high complex modulus but can slip past one another and thus are observed to be fluid rather than solid. A stronger cross-linker, such as α-actinin from chicken smooth muscle, will cross-link the gel into an isotropic network, and the gel will behave as a solid. At higher concentrations of cross-linker, bundles are thermodynamically favored and if the cross-linker can

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dissociate, the filaments can rearrange into bundles. Thus, at high cross-linker concentrations, the gel becomes more fluid-like, while still resisting deformation.

Here we show that biotin-actin/avidin gels are fundamentally different from a tightly cross-linked actin gel made of smooth muscle α-actinin and actin. The biotin-actin/avidin gels are solid at all frequencies, and high concentrations of avidin do not increase the stiffness of the gel. The smooth muscle α-actinin/actin gels are less stiff, and are solid only at low concentrations. This concentration dependence is due to the fact that the α-actinin binding to actin, while strong, is not permanent; the smooth muscle α-actinin dissociates from actin filaments with a rate constant of 0.66/s. The filaments can still rearrange into bundles as they polymerize, and these bundles can behave like a viscoelastic fluid (Wachsstock et al., 1993). A much weaker cross-linker such as amoeba α-actinin, with a dissociation rate constant of 5.25/s, shows a dramatic frequency dependence which can be modeled by the rapid rearrangement of cross-links during deformation.

MATERIALS AND METHODS

Protein purification

Actin from rabbit skeletal muscle and α-actinin from *A. castellanii* were purified as described by Maciver et al. (1991). α-Actinin from chicken smooth muscle was purified as described by Craig et al. (1982). Both α-actinins were dialyzed into Buffer G (2 mM Tris, pH 8.0, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.3 mM NaN_3) before use. These proteins were stored in Buffer G at 4°C and used within 1 week of purification. Avidin was purchased from Pierce Chemical Co. (Rockford, IL) as a lyophilized powder and dissolved at 2 mg/ml in 10 mM Tris, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.3 mM NaN_3 before use.

Fluorescently labeled chicken smooth muscle α-actinin actin-binding domain

The actin binding domain of smooth muscle α-actinin was purified by a modification of the method of Pavalko and Burridge (1991). Briefly, chicken smooth muscle α-actinin (20 ml at about 3 μM) was vacuum dialyzed and concentrated to about 2 ml in Buffer G in a MicroProDiCon (Biomolecular Dynamics, Beaverton, OR). It was then incubated with 25 mg of agarose-linked thermolysin (Sigma Chemical Co., St Louis, MO) for 6 h at 37°C. The thermolysin agarose was removed by filtration through a Poly-Prep column (Bio-Rad, Hercules, CA) and the digested protein loaded on a 5-ml Econo-Q column (Bio-Rad) equilibrated with Buffer G. Under these conditions, the actin binding domain flows through the column and the rod domain is retained.

Rhodamine-labeled actin binding domain was prepared by dialyzing against labeling buffer (10 mM imidazole, pH 7.5, 1 mM EDTA, 0.3 mM NaN_3) overnight at 4°C and adding a 10-fold molar excess of 25 mM iodoacetyltetramethylrhodamine (Molecular Probes, Eugene, OR) in dimethylformamide. The solution was rotated slowly in the dark at 4°C overnight, and free dye was removed by gel filtration over Sepharose G-25 (Sigma). The protein was then digested with thermolysin as above.

Modification of actin

Iodoacetyl-*N'*-biotinhexenediamine (Pierce Chemical Co.) was used to label actin by a modification of the method of Janmey et al. (1990), the same method commonly used to label actin with iodoacetylpyrene (Kouyama and Mihashi, 1980). Briefly, 24 μM non-gel-filtered actin (Spudich and Watt, 1971) was dialyzed into 0.1 M KCl, 2 mM $MgCl_2$, 25 mM imidazole, pH 7.5, 0.3 mM ATP, 0.3 mM NaN_3 and reacted with a 7-fold molar excess of iodoacetylbiotin (40 mM in dimethylformamide). The solution was rotated slowly in the dark at 4°C overnight, and the resulting biotinylated polymerized actin was purified by centrifugation, depolymerization, and gel filtration as described above. The extent of biotinylation was measured by the displacement of 2-(4'-hydroxyazobenzene)benzoic acid from avidin, using a kit from Pierce Chemical Co. (Rockford, IL). The actin used in the experiments described here was 30% biotinylated, and diluted to 2% biotinylated with unlabeled actin.

Rhodamine-labeled F-actin was prepared with iodoacetyltetramethylrhodamine using the same method as for biotinylation.

Rheology

Quantitative physical measurements were made with an R18 cone and plate Weissenberg rheogoniometer (Sangamo Controls, Bognor Regis, Sussex, England) in the forced oscillation mode as described by Sato et al. (1985). The amplitude of oscillations for all experiments was 8 μm, for a maximum shear strain of 1%. Protein samples were mixed and polymerization initiated by adding 10× polymerization buffer to give final concentrations of 2 mM Tris, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA. The sample was immediately applied to the plates of the rheogoniometer and incubated overnight without shearing at 25°C in the instrument. The magnitude of the complex modulus, $|G^*|$, was calculated as $|G^*| = (G'^2 + (2\pi f \eta')^2)^{1/2}$ (Ferry, 1980) where G' is the dynamic shear storage modulus, η' is the dynamic viscosity and f is the frequency of oscillations in Hertz. The phase angle, δ, was calculated as $\tan \delta = 2\pi f \eta'/G'$. The magnitude of the complex modulus was reproducible to within a factor of 5 and the phase shift within 10% in repetitions of the rheological experiments.

Kinetics of polymerization

Polymerization was measured at 25°C by the increase in 90° light scattering at 395 nm. Samples of 1.08 ml were mixed in a plastic cuvette and polymerization initiated by adding 120 μl of 10 × polymerization buffer.

Determination of equilibrium dissociation constants

Mixtures of actin with the actin binding domain of α-actinin were copolymerized and centrifuged as previously described (Wachsstock et al., 1993). Uncentrifuged and supernatant samples were electrophoresed and scanned in a densitometer. The uncentrifuged samples were used to plot a standard curve to determine the free protein concentration. In any given experiment, either the actin or the actin-binding domain concentration was kept constant and used as the concentration of sites in the equilibrium binding equation, bound/sites = free/(K_d + free). The data were fit with the least-squares fitting program, Regression (Blackwell Scientific, Oxford).

Fluorescence anisotropy stopped flow

The equilibrium fluorescence anisotropy of rhodamine-labeled actin-binding domain was measured in an SLM-4800 fluorescence spectrophotometer (SLM Instruments, Urbana-Champaign, IL). Briefly, in a fluorescence anisotropy measurement, the sample is excited with polarized light, and the ratio of parallel and perpendicular polarized emitted light is measured. If the fluorophore is rigid, then the emitted light should be polarized in a fixed direction relative to the incident light. If the fluorophore is rotating more rapidly than the decay time of the fluorescence, the emitted light will be randomly polarized. The anisotropy is $A = A_0/(1 + \tau_f/\tau_r)$, where A_0 is the anisotropy of the immobile fluorophore; τ_f is the fluorescence decay time, about 5 ns for rhodamine (Haugland, 1985); and τ_r is the correlation time for the rotation (Cantor and Schimmel, 1980). Thus, rhodamine labeled actin-binding domain, with a Stokes' radius of 2.3 nm (see below), has a

calculated rotational correlation time of 12 ns (Cantor and Schimmel, 1980) and an anisotropy of $A_0/1.42$. The translation diffusion of actin filaments is near zero (Tait and Frieden, 1982) and filaments 5 μm long (Burlacu et al., 1992), have a calculated rotational correlation time of 55 μs (Cantor and Schimmel, 1980). Thus, a protein bound to an actin filament should be virtually rigid on the time scale of fluorescence decay and should have an anisotropy close to A_0. The fraction of actin-binding domain that is bound to actin filaments is proportional to the increase in anisotropy.

Stopped-flow measurements were made in the instrument previously described (Sinard and Pollard, 1990), modified to have an incident vertical polarizing filter (Melles-Griot, Rochester, NY) and a 540-nm short-pass filter (Oriel, Stratford, CT) and an emission vertical polarizing filter (Melles-Griot, Rochester, NY) and a 570-nm long-pass filter (Oriel, Stratford, CT). The instrument can only measure one emitted light beam, so the true anisotropy could not be measured. Instead, we measured only the parallel component of the emitted light, which increases when the fluorophore stops rotating, as described above.

To measure the association of α-actinin with actin filaments in the stopped-flow machine, polymerized actin in polymerization buffer with Buffer G was mixed 1:1 with actin-binding domain in Buffer G. For dissociation reactions, actin and rhodamine-labeled actin-binding domain were copolymerized in polymerization buffer with Buffer G. The complex was mixed with five parts of this buffer to initiate dissociation. 500 data points at a sampling interval of 0.1 ms for association and 1 ms for dissociation were collected.

Determination of Stokes' radii

We determined the diffusion constants for both molecules using the formula $D = kT/6\pi\eta r$, where k is Boltzmann's constant, T is the absolute temperature, η is the viscosity of water, and r is the Stokes' radius of the molecule. The Stokes' radius was determined by gel filtration on Sephadex G-100 (Sigma) for the actin-binding domain and Sephacryl S-300 for α-actinin, using the method of Seigel and Monty (1966). Myoglobin, carbonic anhydrase, bovine serum albumin, β-amylase, and apoferritin (Sigma) were used to calibrate the columns.

Theoretical modeling

We used the kinetics simulation program HopKINSIM (Wachsstock and Pollard, 1994) to estimate the rate constants for the binding of the α-actinin actin-binding domain to actin filaments, which was assumed to be a simple one-step bimolecular reaction. The rate constants were constrained to maintain the calculated equilibrium dissociation constant equal to that found in the pelleting experiments. The increase in the parallel component of the emitted light is proportional to the fraction of actin-binding domain bound to actin. The coefficient was estimated by calculating the concentration of α-actinin bound at equilibrium from the known K_d, and setting that equal to the observed increase at 1 s, when the reaction is essentially complete.

We used the approach of Shoenberg (1985; also Brenner, 1989; Brenner, 1991) to interpret the rheological data in molecular terms. He showed that a dynamically cross-linked polymer has a stiffness that depends on the velocity of deformation. The force due to the cross-linker in a step deformation of length d is (Shoenberg (1985), Eq. 11) $F = n_B K d e^{-kt}$, where $n_B K$ is the force of the permanently cross-linked network, k is the dissociation rate constant, and t is the time from the deformation. For a two-headed cross-linker like α-actinin, the exponent is $-2kt$. An oscillatory motion such as we use in the rheology experiments is a deformation $d = d_0 \sin \omega t$, where d_0 is the maximum deformation and ω is the frequency of deformation in radians per second. Assuming that superposition is valid, the force equation can be integrated to $F = n_B K d_0 \omega[e^{-2kt}(-2k \cos \omega t + \omega \sin \omega t) + 2k]/(4k^2 + \omega^2)$. At maximum displacement, $t = \pi/2\omega$, and the force is $F = n_B K d_0 \omega(\omega e^{-k\pi/\omega} + 2k)/(4k^2 + \omega^2)$. The chord stiffness is the force divided by the displacement, $S = F/d_0 = S_0\omega(\omega e^{-k\pi/\omega} + 2k)/(4k^2 + \omega^2)$, where S_0 is the stiffness of a permanently cross-linked network. We used this theoretical equation to fit the amoeba α-actinin rheological data.

This model was developed specifically for myosin crossbridges in muscle. It assumes that all of the time dependence of the force relaxation is due to the rate of dissociation of the cross-linker. This is a very restrictive assumption, and will not be entirely valid in the actin system where some of the time dependence is due to the viscous properties of the filaments and filament bundles themselves (Zaner and Hartwig, 1988; Wachsstock et al., 1993). However, the modulus of actin filaments alone is far less dependent on frequency than actin with amoeba α-actinin, and this difference is presumably due to the cross-linker. Any other cause of frequency dependence is explicitly excluded from the model. The validity of this assumption is supported by the agreement to the experimental data (Fig. 5) but needs further testing.

RESULTS

We used a step-by-step approach to understand the mechanical properties of actin filaments with each of the three cross-linkers. First, we show that avidin/biotin cross-linking, like α-actinin cross-linking (Wachsstock et al., 1993), does not affect the kinetics of polymerization of actin (Fig. 1). Second, we determine the rheological properties of biotin/avidin cross-linked actin filaments and show that they are consistent with those of a permanently cross-linked network (Fig. 2, *A* and *B*). Third, we determine the rheological properties of actin filaments cross-linked by amoeba α-actinin (Fig. 2, *E* and *F*) and show that they are consistent with a dynamic network with a dissociation rate constant of 5.25 s^{-1} (Fig. 3). Fourth, we determine the rheological properties of actin filaments cross-linked by chicken α-actinin (Fig. 2, *C* and *D*) and determine the dissociation rate constant to be 0.66 s^{-1} (Fig. 4).

Kinetics of polymerization

Biotinylation of actin and polymerization in the presence of 0.3 μM avidin do not affect the time course or extent of polymerization of 15 μM actin (Fig. 1). The time to half-polymerization is 100 s, and there is a lag of approximately

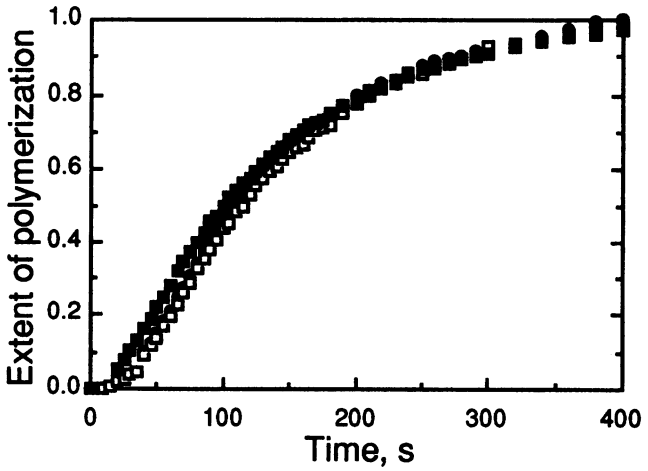

FIGURE 1 Time course of actin polymerization, measured by 90°C light scattering at 395 nm. The time course of polymerization is essentially identical for all three samples: 15 μM unlabeled actin (□), 2% biotinylated actin (●), and 2% biotinylated actin with 0.3 μM avidin (■).

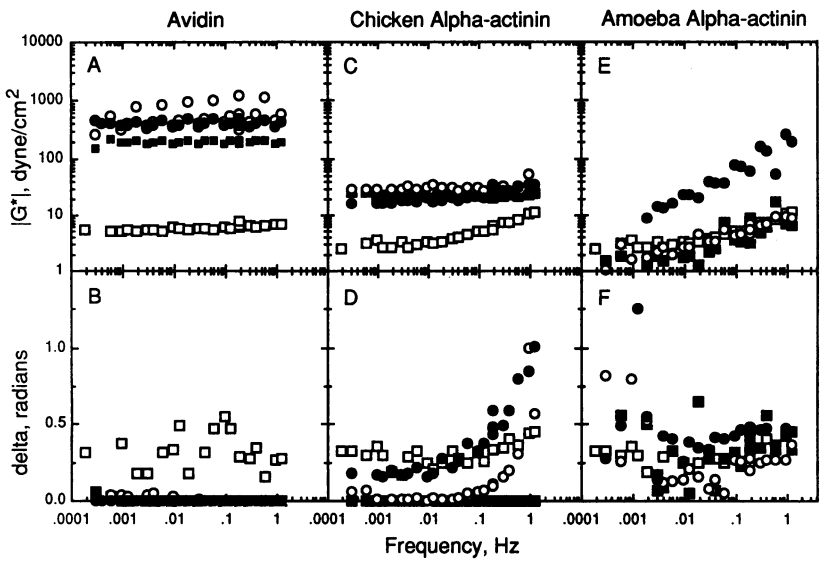

FIGURE 2 Frequency dependence of the rheological properties of actin filaments alone and with varying concentrations of cross-linkers. Actin and cross-linker were polymerized together in the rheogoniometer. (*A*, *C*, *E*) Magnitude of the complex modulus. (*B*, *D*, *F*) Phase shift of the complex modulus. (*A*, *B*) 15 μM, 2% biotinylated actin alone (□) or with 0.03 μM avidin (■), 0.1 μM avidin (○), 0.3 μM avidin (●). (*C*, *D*) 15 μM actin alone (□) or with 0.03 μM chicken smooth muscle α-actinin (■), 0.1 μM chicken α-actinin (○), 0.3 μM chicken α-actinin (●). (*E*, *F*) 15 μM actin alone (□) or with 0.1 μM *Acanthamoeba* α-actinin (■), 0.3 μM amoeba α-actinin (○), 1.0 μM amoeba α-actinin (●). The biotin/avidin increases the magnitude and reduces the phase shift at all frequencies and concentrations. The chicken α-actinin increases the magnitude at all concentrations but not as much, and reduces the phase shift only at certain concentrations. The amoeba α-actinin does not reduce the phase shift and increases the magnitude of the complex modulus only at high concentrations and high frequencies.

20 s as nucleation limits the rate of assembly. This result is strong evidence that biotinylation and avidin have little or no effect on the distribution of filament lengths. It makes it unlikely that either the biotin or avidin nucleates or shortens the filaments. It is known that pyrene labeling actin on cysteine-374 by this method does not affect its polymerization (Cooper et al., 1983; Kouyama and Mihashi, 1980).

Rheology of biotin-actin/avidin and actin/α-actinin gels

The magnitude of the complex modulus of a material measures its resistance to an oscillatory deformation as a function of the amplitude of deformation, while the phase shift between the deformation and the response depends on whether the material is solid or fluid. A solid will resist most when the extent of deformation is maximal, and so the stress will be in phase with the deformation. Such a response is elastic; the material will return to its original shape after the stress is removed. For example, the modulus of steel is 7.6×10^{11} dyne/cm^2, with a phase shift of 0. In contrast, a fluid resists most when the rate of deformation is maximal, so the phase shift is 90° or approximately 1.6 radians. Such a response is viscous; the energy of deformation is lost and the material does not return to its original shape. For example, the modulus of water ranges from 1.2×10^{-2} dyne/cm^2 at 0.19 Hz to 1.9×10^{-6} dyne/cm^2 at 0.0003 Hz, with a phase shift of 1.6 radians. Long tangled polymers are viscoelastic; they will show some recovery and have phase shifts between that of a solid and a fluid. Highly cross-linked rubber has a phase shift of 0.2 radians with a modulus of about 10^8 dyne/cm^2 (Ferry, 1980).

Avidin and the α-actinins differ both quantitatively and qualitatively in their effects on the mechanical properties of actin filament gels (Fig. 2). Actin filaments (*open squares*) have a modulus that varies between 2 and 10 dyne/cm^2 depending on the frequency of deformation, with a phase shift about 0.3 radians (Figs. 2, *C*, *D*, *E*, *F*, *open squares*). Biotinylated actin (Figs. 2, *A* and *B*, *open squares*) has rheological properties very similar to those of pure actin.

Avidin has a very high affinity for biotin (K_d approximately 10^{-15} M (Green, 1990)), close to that of a covalent bond, so actin filaments cross-linked by avidin are permanently cross-linked (Janmey et al., 1990). The magnitude of the complex modulus of such gels is very high (Fig. 2*A*, *filled symbols* and *open circles*). The phase shift of these gels is zero at all frequencies (Fig. 2 *B*). This is the behavior expected of an elastic solid. The elasticity is due to the bending of the filaments, not to any rearrangement of the gel.

Chicken smooth muscle α-actinin binds relatively tightly to actin filaments with an intermediate affinity, a K_d of 0.6 μM (Meyer and Aebi, 1990; Wachsstock et al., 1993). It increases the magnitude of the complex modulus (Fig. 2 *C*), but not by as much as avidin. At these concentrations, the number of cross-links per filament is the same for avidin and chicken α-actinin; the difference in the magnitude of the modulus is due to the ability of the filaments to slip past one another when cross-linked by the weaker α-actinin. The

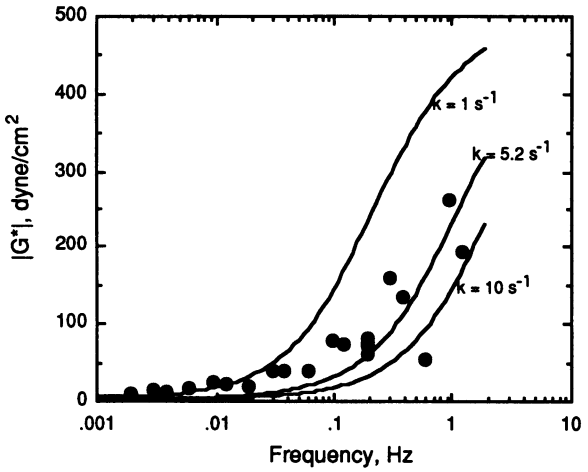

FIGURE 3 Theoretical analysis of the complex modulus of amoeba α-actinin/actin gels. The circles are the data for 1.0 μM amoeba α-actinin and 15 μM actin from Fig. 2E, replotted on a semilogarithmic scale to better show the range of data. The central solid line is the best fit of the dynamic cross-link model to the data, with a dissociation rate constant of 5.2 s^{-1}, as described in the text. The fit is to the original data, not to the logarithmic transformation shown here. The other lines represent the theoretical curves generated for dissociation rate constants of 1 and 10 s^{-1}.

phase shift is zero for 0.03 μM chicken α-actinin with actin, like avidin, but exceeds that of actin alone in the presence of higher concentrations of α-actinin (Fig. 2 *D*). As reported previously (Wachsstock et al., 1993), this is due to the formation of bundles at high concentrations of α-actinin. These bundles can slip past one another more freely than a continuously cross-linked network, making the sample more fluid. The formation of bundles is only possible, because the α-actinin is a dynamic cross-linker and the filaments can diffuse even after an α-actinin molecule cross-links them.

Acanthamoeba α-actinin binds relatively weakly to actin filaments, with a K_d of 5 μM (Wachsstock et al., 1993). A higher concentration of amoeba α-actinin is required to affect the magnitude of the complex modulus (Fig. 2 *E*), because at a given concentration less of the amoeba α-actinin binds to the filaments. The phase shift is relatively high in the presence of amoeba α-actinin (Fig. 2 *F*), because bundles form even at low α-actinin concentrations (Wachsstock et al., 1993). This is attributed to the rapid dissociation of the amoeba α-actinin, allowing the filaments to rearrange and form bundles.

As reported previously (Maciver et al., 1991; Sato et al., 1987; Wachsstock et al., 1993) amoeba α-actinin raises the modulus at high frequencies of deformation, with only a small effect at low frequencies. This was interpreted as an effect of a high dissociation rate constant. Actin networks cross-linked by amoeba α-actinin resist rapid deformations, but at low rates of deformation the networks are similar to actin filaments alone, since the cross-links can rearrange faster than the rate of filament displacement.

To interpret this data more quantitatively, we used a modification of a model previously used to predict the rheological properties of myofibrils (Brenner, 1989, 1991; Shoenberg, 1985); see the Materials and Methods for details. The model assumes that each cross-linker molecule has two independent actin binding sites that can bind actin filaments, be stretched into a high-energy configuration, then relax by dissociating and rebinding in a less strained configuration. It also assumes that each cross-linker binds actin independently of the other, and that the dissociation rate is independent of the strain. We assumed the actin filaments cross-linked with avidin had a stiffness equal to S_0, the stiffness of a permanently cross-linked network. The stiffness S, used in the theoretical analysis was assumed to be equal to the magnitude of the complex modulus less the modulus of the actin filaments alone.

A dissociation rate constant of 5.2 s^{-1} for the complex of *Acanthamoeba* α-actinin with actin filaments gave the best fit of the data with the theoretical equation by nonlinear least square fitting gives (Fig. 3). With the previously measured equilibrium dissociation constant of 4.76 μM (Wachsstock et al., 1993), this gives an association rate constant of 1.1 × 10^6 M^{-1} s^{-1}, which is at the low end of the range for a diffusion-limited reaction. This is expected because one of the reactants, the actin filament, does not diffuse significantly (Doi and Edwards, 1986; Tait and Frieden, 1982), and the other, α-actinin, is a large rod-shaped molecule that diffuses slowly (Loftus, 1988 and this work).

The fitted curve underestimates the complex modulus at low frequencies because the α-actinin molecules in this experiment are under strain, and one would expect that the strain would affect the thermodynamics of the interaction between actin and α-actinin. The affinity should be weaker where the strain is large. This change in energy of binding will be reflected in a change in the dissociation rate constant. Thus, there is really a range of dissociation rate constants in the sample, and this spreads the frequency response and makes it less steep than predicted.[1]

Kinetics of binding of α-actinin to actin filaments

Chicken smooth muscle α-actinin binds actin filaments too tightly to show the sort of frequency dependence observed for the amoeba α-actinin (Fig. 2). We were able to measure the rate of association and dissociation for the smooth muscle α-actinin for comparison with the values calculated for the amoeba α-actinin from the rheological data. The whole α-actinin can bind two actin filaments simultaneously, making analysis of the kinetics of binding very complicated, but smooth muscle α-actinin can be proteolytically cleaved into a rod domain and two actin-binding domains. We assumed that the rate of association of the actin-binding domains with actin filaments is the same as that of the whole α-actinin molecule, after correcting for the diffusion constants and the radii of interaction of the different molecules (Berg and von Hippel, 1985).

We measured equilibrium binding by pelleting and by fluorescence polarization anisotropy (Fig. 4 *A*), and the bind-

[1]We thank Bernhard Brenner of the University of Ulm for this insight.

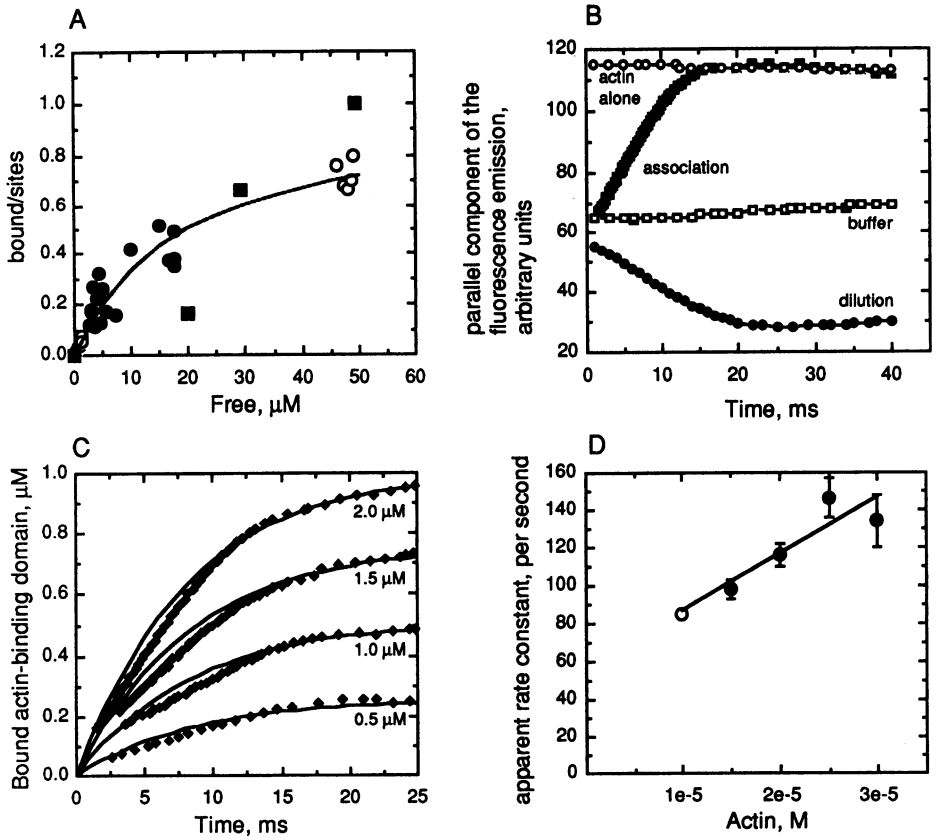

FIGURE 4 Determination of the association and dissociation rate constants for the actin binding domain of chicken α-actinin. (*A*) Determination of the equilibrium binding constant. Actin and actin-binding domain were polymerized together and centrifuged at 120,000 *g*. Identical aliquots of uncentrifuged and supernatant samples were run on a 12% gel, scanned, and bound and free fractions determined as described under Materials and Methods. Closed circles are 20 μM F-actin and varying unlabeled actin-binding domain. Open circles are 50 μM F-actin and varying rhodamine-labeled actin-binding domain. Closed squares are bound and free determinations from anisotropy measurements of 0.5 μM rhodamine-labeled actin-binding domain and varying F-actin, as described in the text. Curve is the best fit to the data, with a K_d of 19.5 μM. (*B*) Typical stop-flow experiments. Open circles are the parallel component of the fluorescence emission for 40 μM F-actin, 10% rhodamine-labeled, mixed one to one with Buffer G. Open squares are the fluorescence of 4 μM rhodamine-labeled actin-binding domain mixed with Buffer G. Closed squares are the fluorescence of 4 μM rhodamine-labeled actin-binding domain mixed one to one with 40 μM F-actin. Closed circles are the fluorescence of 6 μM rhodamine-labeled actin-binding domain copolymerized with 60 μM F-actin and diluted one to five into Buffer G. (*C*) Time course of association of the actin-binding domain to actin filaments and computer simulation of the reaction. Fluorescence units were converted into concentrations of bound actin-binding domain by estimating the equilibrium concentration of bound protein from the equilibrium constant in Fig. 4 *A* and equating that to the final fluorescence intensity in the stop flow experiment. 40 μM F-actin was mixed one to one with varying concentrations of actin-binding domain. The concentrations labeling the curves are the total final concentration of actin-binding domain. The solid lines are theoretical curves calculated with HopKINSIM for a simple bimolecular binding with an association rate constant of 3×10^6 M^{-1} s^{-1} and a dissociation rate constant of 57 s^{-1}. (*D*) Calculated rate constants for the reaction of actin-binding domain to actin with varying actin concentrations. Stop flow experiments with varying actin and actin-binding domain concentrations were performed and fit to exponentials with Cricket Graph (Computer Associates, San Jose, CA), and the apparent rate constant, k_{obs}, plotted as a function of the actin concentration. Error bars are the standard error of the mean for experiments with constant actin concentration and varying actin-binding domain concentration. Solid symbols are association experiments; open symbol represents dissociation experiments. The line is the theoretical line for the rate constants from Fig. 4 *C*; it is not the best fit through the points.

ing rate constants by stopped-flow fluorescence polarization (Fig. 4 *B*). The best fit to the pooled equilibrium data gave an equilibrium dissociation constant for the actin-binding domain of 19.5 μM (Fig. 4 *A*). In the stopped-flow experiments (Fig. 4 *B*), fluorescently labeled actin-binding domain was excited with polarized light. The small freely rotating protein emits unpolarized light, but when it is rigidly bound to an actin filament it will mostly emit light polarized parallel to the incident beam. The intensity of the parallel polarized component is then a measure of what fraction of the actin-binding domain is bound to actin filaments. The interaction of the actin-binding domain with actin can be closely modeled with rate constants of 3×10^6 M^{-1} s^{-1} for association and 57 s^{-1} for dissociation (Fig. 4, *C* and *D*).

The association rate constant for most biological reactions is proportional to the sum of the diffusion constants for the reactants (Berg and von Hippel, 1985; Northrup and Erikson, 1992). The constant of proportionality is a function of the geometry of the interaction. For the purpose of comparing the interaction of actin with α-actinin to that of actin with the actin-binding domain, the geometric factors should be the same, since the same part of the α-actinin molecule is interacting in each case. Actin filaments have a very small diffusion constant (Tait and Frieden, 1982), so the associa-

tion rate constant is proportional to the diffusion constant of the other reactant. This can be calculated from the Stokes' radius, as described under Materials and Methods. We measured the Stokes' radius of α-actinin to be 7.4 nm by gel filtration chromatography, and hence the diffusion constant is 2.94×10^{-7} cm^2/s. Similarly, the Stokes' radius of the actin-binding domain is 2.4 nm and the diffusion constant is 9.2×10^{-7} cm^2/s. The estimated association rate constant for chicken α-actinin to actin is thus 1.20×10^6 M^{-1} s^{-1}. Using the previously measured equilibrium constant for chicken α-actinin of 0.55 μM (Wachsstock et al., 1993) the apparent dissociation rate constant is 0.66 s^{-1}.

DISCUSSION

Most actin cross-linkers, including the α-actinins and ABP-280, have dissociation equilibrium constants in the range of 0.1 to 10 μM. Chicken smooth muscle α-actinin has a K_d of 0.55 μM; amoeba α-actinin has a K_d of 4.7 μM (Wachsstock et al., 1993); macrophage ABP-280 has a K_d of 1 μM (Gorlin et al., 1990). Assuming the association is approximately diffusion-limited (Berg and von Hippel, 1985), the dissociation rate constants for these cross-linker-actin complexes will be in the range of 0.1–50/s, so the cross-links can rearrange in biologically relevant time scales. This sort of dynamic cross-linking may allow the cytoskeleton to rearrange in response to internal or external forces without requiring active signals to sever or polymerize filaments. On the other hand, static cross-linkers such as covalent linkages or biotin/avidin will cross-link the gel into an isotropic solid with no ability to react passively; they can only deform in response to external forces by being taken apart and rebuilt. Bending of polymers allows elastic deformation; when the deforming force is removed, the cell returns to its previous configuration.

The quality of this passive reaction can be regulated by changing the nature of the cross-links or the actin filaments. The length of the filament can be controlled with severing and capping proteins (Weeds and Maciver, 1993). Shorter filaments diffuse more rapidly and are more likely to form bundles (Maciver et al., 1991) so a gel of short filaments will behave as a fluid, not a solid. The affinity of the cross-linker affects the properties of the gel (Wachsstock et al., 1993 and this work). This affinity could be controlled to allow locomotion or cytokinesis. For example, the affinity of many cytoplasmic α-actinins can be controlled by the calcium concentration (Blanchard et al., 1989). The physical properties of the cytoskeleton can also be controlled by the concentration of the actin and cross-linker (Wachsstock et al., 1993), by polymerizing new actin filaments, elongation of actin filaments, or recruiting new cross-linking proteins (Janmey et al., 1990; Janson et al., 1992; Stossel, 1984).

We show here that actin gels cross-linked by α-actinin do not resemble statically cross-linked gels. Actin filaments with the weak cross-linker *Acanthamoeba* α-actinin are viscoelastic fluids (Fig. 2, *E* and *F*). The magnitude of the complex modulus depends on the frequency of the deformation, as is predicted for a dynamic cross-linker that can exchange on the time scale studied (Shoenberg, 1985) and this can be used to estimate the dissociation rate constant.

As shown previously (Wachsstock et al., 1993), actin filaments with chicken α-actinin can either behave as a solid or as a viscoelastic fluid. At low concentrations of α-actinin the mixtures are solids, as Janmey et al. (1990) found for ABP-280. At higher concentrations mixtures are more fluid. At the concentrations of α-actinin tested, the stiffness remained approximately the same. With higher concentrations of α-actinin, the modulus increases (Wachsstock et al., 1993).

An independent method for measuring the kinetics of binding, using stopped-flow fluorescence anisotropy (Fig. 4 and the analysis under Results), gives an association rate constant for chicken α-actinin to actin very similar to that estimated for amoeba α-actinin from the rheological data. This implies that the binding reactions of the two proteins are very similar and that their differences are due to 10-fold difference in their rates of dissociation. The higher affinity of the smooth muscle α-actinin for actin has two effects on the frequency response. First, it shifts the point of maximum frequency dependence to a lower rate. Second, the filaments in amoeba α-actinin/actin gels are bundled at these concentrations and thus the cross-linkers are aligned with one another, similar to the muscle situation for which the quantitative analysis was developed. The smooth muscle α-actinin/actin gel is isotropic, and the molecules are randomly arranged and experience widely varying strains (Wachsstock et al., 1993). This variation in strain flattens the frequency dependence curve and make the quantitative analysis uninterpretable.

The biotin-actin/avidin gels are permanently cross-linked. They are solid at all frequencies and concentrations, and their mechanical properties are independent of frequency in the range tested. If the frequency dependence of amoeba α-actinin/actin gels were due to bending of the filaments, as suggested by Janmey et al. (1988), then it should have been even more pronounced in the static cross-linker case. Therefore, filament bending and breaking do not determine the physical properties of α-actinin-cross-linked actin filaments; these are determined by the nature of the cross-links themselves.

ABP-280 is very different from α-actinin. Nonetheless, the ABP-280 data of Janmey et al. (1990) are consistent with a strong dynamic cross-linker that can slowly rearrange. At the low concentrations of ABP-280 they used, the gel behaved like a solid, but with some creep. Zaner (1988) observed a similar effect. They did not look at the frequency dependence of their biotin-actin/avidin gels, but our data is suggestive that any dynamic cross-linker, even a strong one that acts like a solid on a second time scale, is very different from a convalently cross-linked network.

Cells contain a variety of actin filament cross-linkers that bind actin with a range of affinities. The cell can control its shape and resistance to deformation by compartmentalizing the cross-linkers to different parts of the cell. For instance, nonmuscle ABP-280 is found largely in the cortex (Hartwig and Shevlin, 1986; Mittal et al., 1987), while α-actinin is largely in focal adhesions and in actin filament bundles in cultured cells (Burridge et al., 1988; Geiger, 1989). The high affinity but dynamic nature of the ABP-280 cross-links would allow the cell to resist outside forces but still rearrange slowly (Bray et al., 1986; Rappaport, 1967). The lower affinity α-actinin allows the actin polymerized inside the cell to form stiff filament bundles, which act as a very viscous fluid (Jockusch and Isenberg, 1981; Wachsstock et al., 1993). The dynamic nature of α-actinin cross-links in actin gels means that if the cell severs the filaments in only a small part of the cortex, making it more fluid, the cytoplasm can efficiently flow into the gap, allowing locomotion to take place (Cunningham et al., 1992). The calcium-dependent α-actinin of many nonmuscle cells (Blanchard et al., 1989) allows another level of control of the properties of the cell. By varying the affinity of the cross-linker for actin, the cell can change its cytoplasm from a rigid to a dynamic network. This, combined with calcium-dependent severing proteins like gelsolin (Cortese and Frieden, 1990; Newman et al., 1991), may give the cell precise control over motility and organelle movement.

Note added in proof:—A review submitted when this paper was already in press pointed out that the equation for the analysis for Fig. 3 was integrated over one cycle of oscillation. A more accurate analysis would have integrated over infinitely many cycles for a steady-state result, which gives $S = S_0\omega(\omega^2 + k^2)^{-1/2}$. This equation yields an association rate constant for amoeba α-actinin to actin of 1.3×10^6 $M^{-1}s^{-1}$, rather than $1.1 \times 10^6 M^{-1}s^{-1}$. The conclusions of this paper are not significantly altered. We thank the reviewer for his careful reading.

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