Mechanism of Interaction of Acanthamoeba Actophorin (ADF/Cofilin) with Actin Filaments*

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We characterized the interaction of Acanthamoeba actophorin, a member of ADF/cofilin family, with filaments of amoeba and rabbit skeletal muscle actin. The affinity is about 10 times higher for muscle actin filaments (Kd = 0.5 μM) than amoeba actin filaments (Kd = 5 μM) even though the affinity for muscle and amoeba Mg-ADP-actin monomers (Kd = 0.1 μM) is the same (Blanchoin, L., and Pollard, T. D. (1998) J. Biol. Chem. 273, 25106–25111). Actophorin binds slowly (k– = 0.03 μM–1 s–1) to and dissociates from amoeba actin filaments in a simple bimolecular reaction, but binding to muscle actin filaments is cooperative. Actophorin severs filaments in a concentration-dependent fashion. Phosphate or BeF3 bound to ADP-actin filaments inhibit actophorin binding. Actophorin increases the rate of phosphate release from actin filaments more than 10-fold. The time course of the interaction of actophorin with filaments measured by quenching of the fluorescence of pyrenyl-actin or fluorescence anisotropy of rhodamine-actophorin is complicated, because severing, depolymerization, and repolymerization follows binding. The 50-fold higher affinity of actophorin for Mg-ADP-actin monomers (Kd = 0.1 μM) than ADP-actin filaments provides the thermodynamic basis for driving disassembly of filaments that have hydrolyzed ATP and dissociated γ-phosphate.

Actin filaments, in conjunction with other cytoskeletal proteins, are responsible for maintaining the structural integrity of eukaryotic cells. Phagocytosis, cytokinesis, cell motility, and muscle contraction all depend on structures assembled from actin. Although muscle contraction requires stable actin filaments for force generation, much actin-based motility involves active turnover of filaments. A variety of actin-binding proteins influence actin polymerization, steady-state dynamics of monomers and filaments, and the three-dimensional organization of the filamentous meshwork. At the leading edge of motile cells, the Arp2/3 complex of actin-related proteins is thought to initiate polymerization of a network of actin filaments (1, 2) that turn over on a time scale of minutes (3, 4). This requires the filaments to depolymerize much more rapidly than pure actin filaments in vitro. Proteins of the ADF/cofilin family are thought to promote recycling of actin (for review, see Refs. 5 and 6), because they enhance the dynamics of actin filaments in

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‡‡ The abbreviation used is: DTT, dithiothreitol.

Reagents—Materials came from the following sources: Sigma, dithiothreitol (DTT), 1 EDTA, Tris, sodium azide, Me2SO, hexokinase, ATP, ADP, phospholipid, Sephadex G-25 medium; Molecular Probes (Eugene, OR), tri-(2-carboxyethyl)phosphine, tetramethylrhodamine maleimide 5’ isomer; Whatman (Maidstone, United Kingdom), DEAE-cellulose

MATERIALS AND METHODS

1 The abbreviation used is: DTT, dithiothreitol.
Actophorin Binding Actin Filaments

Preparation and Labeling of Actophorins—Wild type and S88C mutant actophorins (23) or from Acanthamoeba (24), and monomeric Ca-ATP-actin was isolated by Sephacryl S-300 chromatography (25) at 4 °C in G buffer (5 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl2, 0.5 mM DTT). Ca-ATP-actin was labeled with tetramethylrhodamine maleimide 5’ isomer and purified (21).

Other Proteins—Actin was purified from rabbit skeletal muscle actin powder (23) or from Acanthamoeba (24), and monomeric Ca-ATP-actin was isolated by Sephacryl S-300 chromatography (25) at 4 °C in G buffer (5 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl2, 0.5 mM DTT). Actin was labeled on Cys-374 to a stoichiometry of 0.8–1.0 with pyrene iodoacetamide (Ref. 26; as modified by Pollard; see Ref. 24). Mg-ATP G-actin was prepared on ice by addition of 0.2 mM EGTA and an 11-fold molar excess of MgCl2 over actin and used within hours. Actin was polymerized by addition of 1.9 (v/v) 103 M polymerized actin in 10 mM MgCl2, 10 mM EGTA, 100 mM Tris-Cl, pH 8. ADF-BeF3-actin filaments were prepared by polymerizing 20 μM Mg-ATP-actin in 0.1 mM KCl, 2 mM MgCl2, 5 mM NaF, 150 μM BeCl2 at room temperature for 4 h (27).

Assays for Interaction of Actophorin with Actin Filaments—Interaction of actophorin with pyrenyl-labeled actin filaments was followed by the change in fluorescence with excitation at 366 nm and emission at 387 nm (7). Interaction of rhodamine-S88C-actophorin with unlabeled actin filaments was followed by the change in fluorescence anisotropy with excitation at 550 nm and emission at 574 nm (21). Data were collected with an Alphascan spectrofluorometer (Photon Technology International, South Brunswick, NJ). Reactions were initiated by mixing actophorin and actin filaments manually, or for kinetics experiments with a hand-driven stopped-flow mixer (Model SFA-12, HI-tech Scientific Ltd., Salisbury, United Kingdom).

Fluorescence Anisotropy—Mixing rhodamine-actophorin with 7 M rhodamine-actophorin plus muscle actin filaments. The noisy curve is experimental data. Smooth curves are the best single exponential fits to the rising phase and falling phase of fluorescence anisotropy. B, dependence of the maximum fluorescence anisotropy on the concentration of actin filaments. Closed circles, 2 μM rhodamine-S88C-actophorin plus Acanthamoeba actin filaments; open circles, 0.5 μM rhodamine-S88C-actophorin plus muscle actin filaments. Solid lines are the best fits of Equation 2 to the data.

RESULTS

Interaction of actin filaments with ADF/cofilin proteins, including Acanthamoeba actophorin, is complicated by several overlapping reactions, including filament binding (31, 14, 16, 17, 7, 9, 19), a structural change in the filament (32), severing the filament (14, 9), and depolymerization of the filament (7, 19). To study these reactions, we used two fluorescence assays: the fluorescence intensity of pyrenyl-labeled actin and fluorescence anisotropy of rhodamine-labeled S88C actophorin. Pyrenyl fluorescence of polymerized actin is about 20-fold higher than actin monomers (26) and is quenched by binding of some ADF/cofilin proteins, including actophorin (7). A complication is that actophorin can change the fluorescence of pyrenyl-actin filaments by three different mechanisms: (i) binding, (ii) conformational change, or (iii) depolymerization. With proper experimental design, these reactions can be separated in time. Fluorescence anisotropy is sensitive only to the size of the diffusing species, so binding of rhodamine-actophorin to an actin filament increases anisotropy more than binding to an actin monomer. The anisotropy of rhodamine actophorin is 0.14 when free and 0.21 when bound to an actin monomer (21), whereas the polarization anisotropy of rhodamine-phalloidin immobilized on an actin filament is about 0.30.2

Reaction of Actophorin with Actin Filaments Followed by Fluorescence Anisotropy—Mixing rhodamine-actophorin with an excess of unlabeled actin filaments results in a biphasic change in fluorescence anisotropy (Fig. 1A). The first phase is an exponential increase from 0.14 ± 0.005 to a maximum of 0.235 ± 0.005. The rate and amplitude of this phase depend on the concentration of actin filaments. The rate constant for the initial increase in fluorescence anisotropy is in the range of 0.03 μM−1 s−1, the same as measured more accurately (due to

FIG. 1. Fluorescence anisotropy assay for interaction of rhodamine-S88C-actophorin with actin filaments. Conditions were as follows: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM MgCl2, 1 mM EGTA, 0.2 mM ATP, 0.2 mM CaCl2, 0.5 mM DTT, and 3 mM NaN3 at 25 °C. A, time course of the change in fluorescence anisotropy upon mixing 0.4 μM rhodamine-S88C-actophorin with 7 μM muscle actin filaments. The noisy curve is experimental data. Smooth curves are the best single exponential fits to the rising phase and falling phase of fluorescence anisotropy. B, dependence of the maximum fluorescence anisotropy on the concentration of actin filaments. Closed circles, 2 μM rhodamine-S88C-actophorin plus Acanthamoeba actin filaments; open circles, 0.5 μM rhodamine-S88C-actophorin plus muscle actin filaments. Solid lines are the best fits of Equation 2 to the data.

Conditions were as follows: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 0.2 mM ATP, 0.2 mM CaCl₂, 0.5 mM DTT, and 3 mM NaN₃ at 25 °C. Fluorescence following mixing 0.6 mM wild type actophorin from the pyrenyl-actin filaments. The rapid decrease in fluorescence on a faster time scale. Noisy curves are experimental traces. Smooth curves are the best fit using model 2 and values in Table I. B, dependence of $k_{en}$ for the fast phase of the fluorescence change on the concentration of pyrenyl-actin filaments: closed circles, Acanthamoeba pyrenyl-actin filaments; open circles, muscle pyrenyl-actin filaments. Inset, time course of the dissociation of actophorin from muscle pyrenyl-actin filaments by competition with unlabeled actin filaments. A mixture of 2 μM of pyrenyl-actin filaments and 0.4 μM of actophorin was preincubated for 5 min and then mixed with 20 μM unlabeled actin filaments to initiate dissociation of actophorin from the pyrenyl-actin filaments. C, time course of the change in fluorescence following mixing 0.6 μM wild type actophorin with 6 μM muscle pyrenyl-actin filaments: dotted line, in presence of 0.2 mM ADP; noisy line, in presence of 0.2 mM ATP. Smooth curves are the best fit using model 2 and values in Table I.

The amplitude of the maximum increase in fluorescence anisotropy after mixing rhodamine-actophorin with unlabeled actin filaments depends on the concentration of filaments (Fig. 1B). A plot of the maximum amplitude versus the concentration of filamentous actin gives a saturation curve that fits Equation 2. At saturating concentrations of actin filaments the anisotropy is 0.235, higher than rhodamine-actophorin bound to actin monomers (21) but lower than expected for an actin filament. The apparent dissociation equilibrium constant from this analysis depends on the source of the actin. The affinity of actophorin for rabbit skeletal muscle actin filaments ($K_d = 0.49 \mu M$) is 10-fold higher than for Acanthamoeba actin filaments ($K_d = 5.6 \mu M$).

**Binding of Actophorin to Actin Filaments Using Fluorescence of Pyrenyl-actin**—Some ADF/cofilin proteins quench the fluorescence of pyrenyl-actin filaments. However, this is far from a simple bimolecular reaction, so the results depend on many variables, including the source of the actin (Fig. 2B), the presence of the γ-phosphate of ATP bound to actin (see Fig. 4), the ratio of the reactants (Figs. 2 and 3), and the presence of ATP or ADP in the buffer (Fig. 2C). These complications arise from major differences in the actin, including different affinities and degree of binding cooperativity, but also from reactions that follow binding, including severing, depolymerization, and repolymerization.

Reaction of actophorin with an excess of pyrenyl-actin filaments in ATP is biphasic: the fluorescence falls rapidly to a value intermediate between that of polymerized and monomeric actin, followed by a slow recovery (Fig. 2, A and C), similar to the two phase change in fluorescence anisotropy of rhodamine-actophorin. Reaction of an excess of actophorin with pyrenyl-actin causes a monotonic decrease in fluorescence to the level of monomeric actin (Fig. 3, A and B).

With excess pyrenyl-actin filaments the rapid initial phase is an exponential decrease in fluorescence (Fig. 2A, inset) at a rate that depends on the concentration of actin filaments (Fig. 2B). Like Carlier et al. (7), we interpret this decrease in fluorescence to be due to binding of actophorin to actin filaments. Plots of the observed rate constant versus concentration of Acanthamoeba actin filaments are linear with a slope (association rate constant, $k_{-1}$) of 0.029 ± 0.003 μM⁻¹ s⁻¹ and a y-intercept (dissociation rate constant, $k_{+1}$) of 0.11 ± 0.005 s⁻¹. The ratio of these rate constants gives a $K_d$ of 3.8 μM for actophorin binding amoeba actin filaments. For muscle actin, the slope ($k_{-1}$) is 0.008 ± 0.0005 μM⁻¹ s⁻¹ and the y-intercept is near zero, too small to estimate accurately by extrapolation.

To measure the rate constant for actophorin dissociation from muscle actin filaments, we carried out a chase experiment (Fig. 2B, inset). We bound actophorin to pyrenyl-actin filaments and then competed it off by adding an excess of unlabeled actin filaments. The pyrene fluorescence increased during the chase with a rate constant of 0.0050 ± 0.0005 s⁻¹, independent of concentration of unlabeled actin filaments. Unlabeled actin filaments chase ADF-1 from NBD-labeled actin filaments at a rate of 0.035 s⁻¹ (19). We interpret this to be the rate of dissociation of actophorin from the pyrenyl-actin filaments. For muscle actin, the $K_d$ calculated from the rate constants is 0.6 μM. Both of these equilibrium constants derived from kinetics agree well with the values from fluorescence anisotropy.

After samples with an excess of pyrenyl-actin filaments reached a minimum fluorescence, the fluorescence recovered slowly in the presence of ATP (Fig. 2, A and C). The fluorescence anisotropy, elaborated upon under “Discussion,” is that the first phase is due to binding of rhodamine-actophorin to actin filaments, whereas the second phase results from severing and depolymerization of filaments.
Actophorin Binding Actin Filaments

Fig. 3. Fluorescence assay of the time course of interaction of pyrene-labeled actin filaments with an excess of unlabeled actophorin. Conditions were as follows: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 0.2 mM ATP, 0.2 mM CaCl₂, 0.5 mM DTT, and 3 mM NaN₃ at 25 °C. A, time course of the change in fluorescence following mixing of 2 μM pyrenyl-amoeba-actin filaments with various concentrations of actophorin: 15, 20, 30, and 40 μM from the top to bottom curve, respectively. B, time course of the change in fluorescence following mixing of 2 μM pyrenyl-muscle-actin filaments with various concentrations of actophorin: 4, 6, 8, 10, 12, and 15 μM from the top to bottom curve, respectively. Inset shows the early time course. Smooth curves are the best fit using model 1. C, dependence of kₜₚ occured on the fluorescence change of the concentration of actophorin. In the absence of phosphate: open circles, muscle actin; closed squares, amoeba actin; and with 68 mM phosphate: closed circles, muscle actin. The solid lines are manual fits to the data points. The data point at zero actophorin comes from Fig. 2B.

Fluorescence increased exponentially with a rate constant of 0.015 ± 0.001 s⁻¹ for amoeba actin (Fig. 2A) and 0.0030 ± 0.0005 s⁻¹ for muscle actin (Fig. 2C). The fluorescence did not recover when the buffer contained ADP rather than ATP (Fig. 2C).

When an excess of unlabeled actophorin was mixed with amoeba pyrenyl-actin filaments (Fig. 3A) or muscle pyrenyl-actin filaments (Fig. 3B), the fluorescence decreased to the level of monomeric actin and did not recover even in ATP. With amoeba pyrenyl-actin filaments (Fig. 3A), the time courses fit single exponentials, and the dependence of kₜₚ on the concentration of actophorin was linear, as expected for a simple bimolecular reaction (Fig. 3C). The slope and intercept give the same rate constants as the experiment with excess filaments (Fig. 2B).

In contrast, the time course of the reaction of an excess of unlabeled actophorin with muscle pyrenyl-actin filaments does not fit a single exponential (Fig. 3B). An initial lag of a few seconds was followed by a progressively faster decrease in fluorescence to baseline level. The lag was most pronounced at low concentrations of actophorin. Both the duration of the lag and the rate of the subsequent decline in fluorescence depend on the actophorin concentration. In contrast to experiments with excess pyrenyl-actin filaments (Fig. 2), the dependence of kₜₚ for the decline in fluorescent after the lag phase on actophorin was not linear (Fig. 3C). As elaborated upon under “Discussion,” these features suggest that binding of actophorin to muscle actin filaments is cooperative.

Inhibition of Actophorin Binding to Pyrenyl-actin Filaments by Phosphate and BeF₃—Maciver et al. (14) found that inorganic phosphate inhibits the ability of actophorin to reduce the low shear viscosity of actin filaments but did not establish whether this was due to inhibition of binding or some subsequent reaction, such as severing or depolymerization. The half maximal effect was at 5–10 mM phosphate, similar to the Kᵣ of 1 mM for phosphate binding to ADP-actin filaments (33). New kinetic experiments with saturating phosphate (68 mM) established that actophorin and phosphate compete for binding muscle ADP-actin filaments (Fig. 4). Mixing an excess of actophorin with muscle pyrenyl-actin filaments in 68 mM phosphate caused a slow exponential decrease in fluorescence to the level of monomeric actin. In contrast to samples without phosphate, the rate is much slower and there is no lag. As in the absence of phosphate, the observed rate constants in 68 mM phosphate vary nonlinearly with actophorin concentration, but the rates are 10 times smaller (Fig. 3C).

BeF₃ binds ADP-actin filaments with an affinity 3 orders of magnitude higher than phosphate (27) and completely inhibits the effect of 20 μM actophorin on the fluorescence of pyrenyl-actin filaments (Fig. 4). The result is similar with filaments stabilized with phalloidin, explaining the ability of phalloidin to protect filaments from actophorin (14). Pelleting experi-
ments confirm that no actophorin binds actin filaments saturated with BeF₃ or phalloidin, ruling out the possibility that actophorin binds without changing the fluorescence of pyrenyl actin.

**Actophorin Increases the Rate of Phosphate Release from Actin Filaments**—The 2-amino-6-mercaptop-7-methyl purine riboside-phosphorylase assay (28, 29) showed that actophorin increases the rate of phosphate dissociation. Without actophorin, phosphate release lags behind polymerization, with a rate constant of 0.0022 s⁻¹ (Fig. 5A, in good agreement with Ref. 29). With a high concentration of actophorin, phosphate release keeps pace with polymerization, so the actual rate of phosphate release is faster than the observed rate of 0.032 s⁻¹ (Fig. 5B). According to kinetic simulations, the minimum rate of phosphate release from filaments saturated with actophorin is 0.04 s⁻¹ and could be much faster. Note that actophorin increases the absorbance change used to follow polymerization. The actophorin concentration dependence of phosphate dissociation suggests a $K_d$ of about 20 μM for actophorin binding ADP-Pi filaments (Fig. 5C). Addition of actophorin to actin filaments just at the completion of spontaneous polymerization induces a burst in phosphate release. In contrast, phalloidin strongly inhibits the rate of phosphate release in agreement with previous work (34).

**Actophorin Severs Actin Filaments**—A kinetic assay for actin filament ends confirms that actophorin severs actin filaments (Fig. 6). The experiment consisted of two steps. First, unlabeled actin filaments were reacted with a range of actophorin concentrations or with a fixed concentration for a range of times. These reaction mixtures were used to seed the polymerization of 5 μM pyrene-muscle-actin monomers. This assay measures the number concentration of filament ends, because the rate of polymerization is proportional to the concentration of ends.
because the concentration of actophorin in the elongation assay is much lower than the concentration of pyrenyl-actin monomers, and because actophorin has little or no effect on the rate of elongation of ATP actin monomers (21). The fact that the extent of elongation is unaffected by actophorin (Fig. 6A) is further evidence that these concentrations of actophorin do not affect the elongation reactions at either end of filaments.

Incubation with actophorin increases the rate of elongation from preformed actin filaments. The increase in elongation rate depends on the duration of preincubation of the preformed actin filaments with actophorin (Fig. 6A and B) and at a fixed time point on the concentration of actophorin (Fig. 6B). We interpret the increase in the rate of elongation as an increase in the concentration of filament ends. Assuming no effect on elongation rate constants, we used Equation 1 to calculate the number concentration of actin filaments from the initial rate of polymerization. The number of muscle actin filament ends increased to a maximum after 5 min and then declined (Fig. 6B). The number of filaments at 5 min increased with the concentration of actophorin up to 10-fold at 4 μM actophorin (Fig. 6B). Maciver et al. (9) carried out a similar experiment with qualitatively similar results with actophorin and human ADF. They observed a transient 5-fold increase in the number of ends after 30 s with equimolar actophorin and muscle actin filaments.

It was more difficult to resolve the time course and measure the concentration of ends in kinetic experiments with Acanthamoeba actin. Ten seconds of preincubation of actophorin with amoeba actin filaments increased the rate of elongation in the second phase of the assay, but the effect was transient. After 600 s, the elongation rate seeded by the mixture of actophorin and amoeba actin filaments was less than the rate of untreated filaments. This difference, considered in more detail under “Discussion,” may be due to faster binding, severing, and depolymerization of Acanthamoeba actin filaments by actophorin.

**DISCUSSION**

**Binding of Actophorin to Actin Filaments**—Mossakowska and Korn (35) and our previous paper (21) were among the first reports comparing the interactions of an ADF/cofilin protein with actins from the same cell and from muscle. Because of its convenience, most laboratories use skeletal muscle actin (7, 9, 12, 16, 17, 19, 20, 36). Two studies of yeast cofilin used yeast actin (37, 10) but did not make a systematic comparison with earlier work with muscle actin (38). Our results show that a full understanding of the physiological properties of ADF/cofilin proteins requires experiments with homogeneous systems.

Although actophorin has the same affinity for actin monomers from muscle and amoeba (21), its interactions with muscle and amoeba actin filaments differ qualitatively and quantitatively. For example, binding to muscle actin filaments is cooperative, but binding to amoeba actin filaments is not. The affinity for muscle ADP-actin filaments is 10 times higher than amoeba actin filaments, due to major differences in both the association and dissociation rate constants. The association rate constant is 3 times higher for actophorin binding amoeba ADP-actin filaments than muscle actin filaments, but actophorin dissociates 20 times faster from amoeba actin. This difference explains why Cooper et al. (18) found that no stable association between actophorin and amoeba actin filaments. We do not know whether other ADF/cofilin proteins interact the same or differently with their own actin and muscle actin.

**Kinetics of the Interaction of Actophorin with Actin Filaments**—Binding of actophorin to actin filaments is complicated by severing and depolymerization of the filaments and influenced by dissociation of the γ-phosphate released by hydrolysis of the bound ATP. Furthermore, binding to muscle actin filaments is cooperative. Fortunately, using a combination of assays and varying the ratios of the reactants, it is possible to separate all of these overlapping events in time. The following sections address each phase of the reaction.

**Simple Binding of Actophorin to Excess ADP-actin Filaments**—This is the most straightforward reaction, with similar results from both fluorescence anisotropy of rhodamine-SSSC-actophorin binding unlabeled actin filaments and unlabeled actophorin binding pyrenyl-actin filaments. Excess of actin minimizes the effect of interactions between actophorins bound near to each other on the same filament. The time course follows a single exponential, and kobs is directly proportional to the concentration of actin filaments, as expected for a bimolecular reaction. The slope gives association rate constants of k$_{1}$ of 0.029 μM$^{-1}$ s$^{-1}$ for amoeba actin ADP-actin filaments and 0.008 μM$^{-1}$ s$^{-1}$ for muscle ADP-actin filaments. The dissociation rate constant for amoeba actin, estimated from the y-intercept, is 0.11 s$^{-1}$, giving the same binding constant as that measured in equilibrium experiments by fluorescence anisotropy (Fig. 1). The ratio of the dissociation rate constant from muscle actin, estimated from a chase experiment (Fig. 2B, inset), to the association rate constant also agrees well with the equilibrium experiment. The affinity is 10 times higher for muscle actin than amoeba actin filaments, due largely to the slower dissociation of actophorin from muscle actin filaments. These association rate constants are remarkably low, orders of magnitude slower than binding of myosin (9 μM$^{-1}$ s$^{-1}$) (39), α-actinin (2.5 μM$^{-1}$ s$^{-1}$) (40, 41), or gelsolin (20 μM$^{-1}$ s$^{-1}$) (42) to actin filaments. To account for the slow association rate, we postulate that bare ADP-actin filaments have few open actophorin binding sites. Phalloidin also binds slowly because filaments rarely sample the conformation with an open binding site between the subunits (43, 44).

**Excess Actophorin Binding to ADP-actin Filaments**—Excess actophorin binds amoeba actin filaments in a simple bimolecular reaction (Fig. 3A), with rate constants the same as in experiments with excess actin. This shows that binding is not cooperative. On the other hand, when an excess of actophorin binds muscle ADP-actin filaments, the reaction is complicated by effects of bound actophorin on subsequent binding reactions, as observed for ADP (19). This positive cooperativity accelerates binding after an initial lag of <1 s (Fig. 3B). Fortunately, an excess of actophorin saturates the filaments and reduces the pyrenyl fluorescence to the level of monomeric pyrenyl-actin, so that subsequent reactions (including severing and depolymerization) do not produce a signal to complicate further the analysis of the association reaction.

The simplest interpretation (19) is that initial slow binding of actophorin accelerates the binding of subsequent actophorins to the same filament. A two-step mechanism (Scheme 1) accounts for our data.

![Scheme 1](http://www.jbc.org/)

This model assumes that initial slow binding of actophorin to muscle actin causes a conformational change in the filament that accelerates further binding of actophorin. C is the concentration of actophorin, F is the concentration of polymerized actin, *F* represents polymerized actin with a conformational change induced by actophorin, and *FC* represents the complex with actophorin. The rate of the conformational change induced
by actophorin is proportional to $k_{-1}$ (rate constant measured for actophorin binding an excess ADP-actin filaments (Fig. 2)) and some power of the concentration of actophorin. The rate constant for actophorin binding to *F is an unknown. The first reaction in Scheme 1 is written as a catalytic, and the concentration of C consumed in forming *F is ignored, because most C is free in this experiment, so the initial slow binding inducing the actin filament conformational change does not affect actophorin concentration.

We varied the two unknowns until computer simulations of the kinetic curves matched the time course of the change in fluorescence over a range of actophorin concentrations (Fig. 3B). The initial lag was best fit when the rate of the first step was equal to the product of the measured association rate constant and the square of the actophorin concentration. Similar to the results of Ressad et al. (19) with plant and human ADF, neither the first nor the third power of the concentration gave good fits. As in their model, the initial binding of two actophorins in close proximity induces a conformational change in the filament that accelerates further binding of actophorin. The subsequent time course was fit best with an association rate constant, $k_{-2}$ of 0.075 $\mu$M$^{-1}$ s$^{-1}$, 10 times larger than the rate constant for actophorin binding bare muscle ADP-actin filaments, but only 2.5 times the rate constant for the noncooperative binding of actophorin to bare amoeba ADP-actin filaments. This mechanism includes the same concepts as a model proposed for plant and human ADF binding to muscle ADP-actin filaments (19), although the rates are substantially lower for actophorin than ADF. The conformational change induced by actophorin may correspond to the change in the twist of the filament described by McGough et al. (32), although additional work is required to prove this point.

This mechanism is attractive for several reasons. First, most subunits in bare actin filaments are in the standard conformation, whereas thermal motion allows a few ADP-subunits to sample the twisted conformation. Second, because binding of ADF/cofilin proteins favors the twisted conformation, then the twisted conformation is likely to favor binding of ADF/cofilin proteins. Thus, the standard conformation may have few sites favoring ADF/cofilin binding, explaining the very slow association rate. Further, binding of a few ADF/cofilin proteins will favor (trap) the twisted conformation, making adjacent sites available for rapid binding, explaining the cooperative binding. If bound ATP, ADP-P$_i$, and phalloidin all favor the standard conformation over the twisted conformation, their inhibition of binding is explained.

This cooperative mechanism applies to ADF/cofilins binding to muscle actin filaments, but is it relevant to physiology? The cooperatively for amoeba actin filaments is far less and binding to bare filaments is nearly as fast as to activated muscle actin filaments. New experiments will be required to learn whether this is a general property of cytoplasmic actin filaments.

**Actophorin Severs Actin Filaments**—Elongation experiments (Fig. 6 and Ref. 9) confirm earlier evidence that actophorin and other ADF/cofilins sever actin filaments. Severing depends on the concentration of actophorin, the type of actin, and time. In 5 min, 4 $\mu$M actophorin severs each filament into about 10 pieces. This is similar to the extent of severing observed by Maciver et al. (9) with actophorin in a similar assay and by Du and Frieden (10) with yeast coflin and actin using photo-bleaching recovery to estimate length. The accumulation of muscle actin filament ends due to severing plateaus after 400 s. Several factors may contribute to the plateau: short filaments may not sever as readily as long filaments (9), or severing may continue but be balanced by reannealing or the disappearance of short filaments, because actophorin does not cap and stabilize severed ends as does gelsolin.

**Reactions Subsequent to Actophorin Binding to Actin Filaments**—Under certain conditions our fluorescence assays revealed additional reactions—severing, depolymerization and repolymerization—follow actophorin binding to actin filaments. We do not understand most of these reactions nearly as well as binding, but we know enough to model them approximately. The slow drop in fluorescence anisotropy (Fig. 1) after binding of rhodamine-actophorin to unlabeled actin filaments indicates that the actophorin either dissociates from the filaments or, more likely, that the ADP-actin to which it is bound is either in a smaller filament (due to severing) or has depolymerized. Neither depolymerization nor severing is apparent when actophorin is in excess over pyrenyl-ADP-actin filaments, because binding alone reduces the pyrene fluorescence to the level of actin monomers (Fig. 3). Thus, neither severing nor depolymerization causes further change. However, with excess actin, fluorescence drops only part of the way to the monomer fluorescence (Fig. 2). In ADP, the fluorescence is steady at this intermediate level, but in ATP the fluorescence recovers slowly toward the level of polymerized actin. Others have observed similar recoveries in pyrene fluorescence (45, 7). Aizawa et al. (45) did not comment, and Carlier et al. (7) suggested that the slow increase might be due to a redistribution of plant ADF from low affinity binding to pyrenyl-actin subunits to high affinity binding to unlabeled actin subunits in the filaments. Our binding experiments using fluorescence anisotropy or pyrene fluorescence show that actophorin has a similar affinity for unlabeled (K$_f$ = 5.9 $\mu$M) and 100% pyrene-labeled (3.8 $\mu$M) actin filaments, ruling out that explanation.

After actophorin binds ADP filaments, the pyrene fluorescence recovers partially in presence of ATP. Our interpretation is that the intermediate fluorescence is due to a steady state that includes accelerated depolymerization of ADP-actin filaments, exchange of ADP on dissociated monomers for ATP and repolymerization of ATP-actin to form filaments that transiently do not bind actophorin owing to their content of ATP and ADP-P$_i$ subunits. To test this hypothesis, we simulated a simple cycle reaction of actophorin with pyrenyl-ADP-actin filaments followed by recovery using the mechanism in Scheme 2, where C is actophorin, A is actin monomer, T is ATP, D is ADP, P is P$_i$, E is filament ends, and F is polymerized actin. Although this scheme appears complicated, most of the rate and equilibrium constants are known (Table I) except for $k_{-6}$ (the rate of dissociation of actophorin-ADP-actin subunits from filaments), $k_{-12}$ (the rate of dissociation of ADP-actin from filaments partially saturated with actophorin), and $k_{-9}$ (the rate of severing).

Simulations of the time course of pyrenyl-actin fluorescence fit the observations remarkably well (Fig. 2, A and C), using the measured rate constants and reasonable values for the number of ends present at the beginning of the experiments (0.6 nm for muscle actin and 2.9 nm for amoeba actin) and estimates of unknown rate constants: $k_{-3}$ = 0.05 s$^{-1}$ and $k_{-12}$ = 40 s$^{-1}$ for amoeba actin; $k_{-9}$ = 0.02 s$^{-1}$ and $k_{-12}$ = 20 s$^{-1}$ for muscle actin. The values of $k_{-9}$ agree well with the rate of severing.
measured directly $k_{-9} = 0.012 \text{ s}^{-1}$ (Fig. 6B). In experiments with an excess of actin filaments over actophorin, most depolymerizing subunits are ADP-actin rather than actophorin-ADP-actin subunits. Consequently, the data do not constrain the value of $k_{-6}$. In agreement with these observations, the simulations show no recovery with ADP. In ATP, dissociating ADP-actin exchanges ADP for ATP, and owing to the low critical concentration for ATP actin, it repolymerizes at the ends of the numerous severed filaments with a concomitant increase of pyrene fluorescence. ATP hydrolysis and phosphate release are slow enough for the polymer concentration to recover partially without rebinding actophorin. The shape of the curves in Fig. 2, A and C, constrains $k_{-12}$ to between 30 and 50 $\text{s}^{-1}$ for amoeba actin and between 15 and 25 $\text{s}^{-1}$ for muscle actin. Varying the value of each of the unknown parameters more than 30% yielded theoretical curves that failed to fit the full set of experimental curves, even if the values of other unknowns were varied in a compensatory fashion.

The estimated rate constants are reasonably robust and agree well with previous work (20, 10). The value proposed for $k_{-12}$ is the same order of magnitude as the rate of depolymerization of ADP-actin subunits from the barbed ends, but more than 1 order of magnitude larger than dissociation from the pointed ends without ADP/cofilin binding. Available evidence suggests that ADP/cofilins promote both severing and depolymerization from pointed ends (7, 19, 20) and perhaps barbed ends as well. Severing and depolymerization reactions still need more work, but the large difference in affinity of actophorin for amoeba ADP-actin monomers relative to ADP-actin filaments provides a thermodynamic basis for severing amoeba actin more effectively than muscle actin filaments, which bind actophorin almost as tightly as muscle actin monomers.

**Phosphate Dissociation as a Timer for Polymer Destruction**—Occupation of the γ-phosphate site in actin filaments by phosph or BeF$_2$ inhibits actophorin binding. This explains how millimolar concentrations of phosphate protect actin filaments from the effects of actophorin on the slow shear viscosity (14, 9) and of ADP1 on binding (4) and depolymerization (7). A lack of actophorin binding to ADP-P$_i$ filaments and phalloidin-ADP filaments in poling assays establishes that the absence of a fluorescence signal is due to a lack of binding rather than failure of bound actophorin to induce a fluorescence change in pyrenyl ADP-P$_i$-actin filaments.

Phosphate and actophorin compete for binding ADP-actin filaments. As expected from detailed balance, BeF$_2$ is a much stronger inhibitor of actophorin binding, because it binds much more tightly to the γ-phosphate position than phosphate. The thermodynamic relationships remain to be determined by further experiments, but the kinetic consequences are already clear: tightly bound BeF$_2$ reduces the rate of actophorin binding more than 2 orders of magnitude, weakly bound phosphate inhibits the rate of actophorin binding more than 10-fold, and actophorin stimulates the rate of phosphate release more than 10-fold. Actophorin binding may change the subunit conformation to favor the proposed “back-door” pathway of phosphate dissociation (46).

Competition between actophorin and phosphate makes binding of excess actophorin to ADP-P$_i$ muscle actin filaments (Fig. 4) qualitatively different from binding to muscle ADP-actin filaments (Fig. 3B). Binding is not only much slower but is also less cooperative. The time course follows a single exponential and the dependence of $k_{-3}$ on actophorin concentration gives an association rate constant of 0.003 $\text{μm}^{-1} \text{s}^{-1}$, three times lower than the initial slow binding of actophorin to excess ADP-actin filaments (Fig. 2). At no concentration did we observe a lag followed by rapid cooperative binding, so overall, the rate of binding is 15 times slower to ADP-P$_i$-actin filaments than ADP-actin filaments at 15 μm actophorin (Fig. 4).

Obviously, the situation during active polymerization in the presence of actophorin is complicated, because not only do ATP hydrolysis and phosphate release regulate actophorin binding, but actophorin affects phosphate release. Further work is required to understand the detailed relationships and their consequences for actin filament severing and depolymerization. At the very least, by enhancing phosphate release actophorin accelerates the cycle of polymerization depolymerization. Phosphorylation of ADP/cofilins by LIM kinase inhibits binding to actin filaments (47, 48)$^3$ and is another potential avenue of regulation.

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Actophorin Binding Actin Filaments

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