Binding of Phosphate, Aluminum Fluoride, or Beryllium Fluoride to F-actin Inhibits Severing by Gelsolin*

(Received for publication, July 24, 1995, and in revised form, November 17, 1995)

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Actin exhibits ATPase activity of unknown function that increases when monomers polymerize into filaments. Differences in the kinetics of ATP hydrolysis and the release of the hydrolysis products ADP and inorganic phosphate suggest that phosphate-rich domains exist in newly polymerized filaments. We examined whether the enrichment of phosphate on filamentous ADP-actin might modulate the severing activity of gelsolin, a protein previously shown to bind differently to ATP and ADP actin monomers. Binding of phosphate, or the phosphate analogs aluminum fluoride and beryllium fluoride, to actin filaments reduces their susceptibility to severing by gelsolin. The concentration and pH dependence of inhibition suggest that HPO$_4$$^{2-}$ binding to actin filaments generates this resistant state. We also provide evidence for two different binding sites for beryllium fluoride on actin. Actin has been postulated to contain two P$_i$ binding sites. Our data suggest that they are sequentially occupied following ATP hydrolysis by HPO$_4$$^{2-}$, which is subsequently titrated to H$_2$PO$_4$$^-$. We speculate that beryllium fluoride and aluminum fluoride bind to the HPO$_4$$^{2-}$ binding site. The cellular consequences of this model of phosphate release are discussed.

The hydrolysis of ATP during actin polymerization has been postulated to alter filament structure (1–3, 4), change the kinetics of monomer binding (5, 6), and modulate the association of actin binding proteins (7–9). Biochemical evidence suggests that ATP hydrolyzes rapidly as monomers add to the end of a filament, but that subsequent release of hydrolysis products is slow (10). In F-actin at steady state, ADP is bound within the filament, but that subsequent release of hydrolysis products is slow. ATP hydrolyzes rapidly as monomers add to the end of a filament, and modulate the association of actin binding proteins (7–9). Biochemical evidence suggests that ATP hydrolyzes rapidly as monomers add to the end of a filament, but that subsequent release of hydrolysis products is slow (10). In F-actin at steady state, ADP is bound within the filament, but that subsequent release of hydrolysis products is slow. ATP hydrolyzes rapidly as monomers add to the end of a filament, but that subsequent release of hydrolysis products is slow (10). In F-actin at steady state, ADP is bound within the filament, but that subsequent release of hydrolysis products is slow.

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**MATERIALS AND METHODS**

BeSO$_4$ and Al(SO$_4$)$_3$ were purchased from Aldrich. Phallolidin, tetramethylrhodamine isothiocyanate (TRITC-phallolidin), NaF, nucleotides, and all other buffers and salts were purchased from Sigma.

 Proteins—Monomeric (G) actin was purified from an acetone powder of rabbit skeletal muscle as described previously (22, 23). G-ADP-actin was prepared by treatment of G-ATP actin with hexokinase-glucose followed by dialysis in G-ADP buffer as described previously (7). Pyrene

The abbreviations used are: F-actin, filamentous actin; G-actin, monomeric actin; PIA, pyrene iodoactamide; P$_i$, inorganic phosphate; ADP-P$_i$, the nucleotide state in which hydrolysis has occurred but release of products has not; AlF$_3$, aluminum fluoride, exact ionic species unknown; BeF$_2$, beryllium fluoride, exact ionic species unknown; s1–3, N-terminal truncate of human plasma gelsolin containing domains 1–3 of the parent molecule; TRITC, tetramethylrhodamine isothiocyanate.
iodacetamide was coupled to ATP-actin as described previously (23). G-ADP-PiAIActin was prepared by treatment of G-ATP-PiAIActin with hexokinase-glucose followed by dialysis for 24–48 h in 2 mM Tris, pH 8.0, 0.5 mM ADP, 0.2 mM CaCl₂, and 0.2 mM dithiothreitol (7). Actin concentrations were determined from the absorbance at 290 nm using an extinction coefficient of 0.62 ml mg⁻¹ cm⁻¹.

F-ADP-actin was polymerized at concentrations of 15 to 30 µm in solutions containing 150 mM KCl, 20 mM HEPES, pH 7.4, 0.5 mM ADP, 0.2 mM dithiothreitol, 2 mM MgCl₂, and 0.2 mM CaCl₂. F-ADP-actin was generated by polymerization of G-ADP-actin in similar solutions containing various concentrations of KH₂PO₄ and K₂HPO₄ (varied to give the appropriate pH) as indicated under "Results," and KCl, with a total K⁺ concentration equal to 150 mM (15). The N-terminal fragment of gelsolin, s1–3, was purified from Escherichia coli as described (25).

Severing Assay—The severing assay is based on the ability of gelsolin and gelsolin-like molecules to displace phalloidin from F-actin. TRITC-phalloidin bound to F-actin has an enhanced emission (26), which is lost when gelsolin displaces the molecule from the filament (27). F-actin was added to solutions containing 1 µM TRITC-phalloidin, and the fluorescence increase upon phalloidin binding measured in a Perkin Elmer LS-50b fluorescence spectrophotometer. The solutions were continuously stirred using the stirrer of the LS-50b on the low setting. Subsequent addition of gelsolin, or gelsolin s1–3 at concentrations of 50 mol % of the actin concentration reduced the TRITC-phalloidin fluorescence to that in the absence of F-actin. The fluorescence signal was normalized to 1.0 using the following equation:

\[ I_{\text{normal}} = \frac{(I - I_{bg})}{(I_{\text{max}} - I_{bg})} \] (Eq. 1)

where \( I_{bg} \) is the fluorescence at time \( t \), \( I_{max} \) is the fluorescence of TRITC-phalloidin in the absence of actin, and \( I_{\text{max}} \) is the fluorescence of the TRITC-phalloidin in F-actin mixture.

While the kinetics of gelsolin severing TRITC-phalloidin-bound F-actin depend on divalent cation concentrations (27) the severing by gelsolin s1–3 was found to be completely independent of the presence of divalent cations, as previously reported (25). For experiments using intact gelsolin, the Ca²⁺ concentration was measured as described previously (27) using Mag Fura 5 (Molecular Probes, Eugene, OR).

RESULTS

Using gelsolin’s ability to displace phalloidin from F-actin and the enhanced fluorescence of TRITC-phalloidin upon binding to actin as a severing assay (27), we investigated whether the binding of Pₚ to F-ADP-actin alters actin’s susceptibility to severing. Reduction of free Ca²⁺ by Pₚ was accounted for by titration of [Ca²⁺] in control buffers with EGTA. Ca²⁺ concentrations were measured using the ratiometric dye Mag Fura 5, as described previously (27).

Fig. 1 demonstrates that binding of Pₚ to F-ADP-actin reduces the extent of severing by human plasma gelsolin. F-ADP-actin was incubated overnight in buffers containing increasing concentrations of Pₚ, diluted in identical buffers containing TRITC-phalloidin and allowed to come to equilibrium (as determined from the increased emission of TRITC-phalloidin). Addition of gelsolin reduced the fluorescence to baseline levels, as previously reported. Gelsolin displaced all the TRITC-phalloidin from F-ADP-actin within 5 min at all but the lowest Ca²⁺ concentration. In contrast, F-ADP-Pₚ-actin showed a Pₚ concentration-dependent resistance to severing by gelsolin within this time scale. This result suggests that binding of Pₚ to F-ADP-actin generates filaments resistant to gelsolin. However, to escape complications from the alterations in free Ca²⁺ by Pₚ, we continued these studies using bacterially expressed N-terminal gelsolin fragment s1–3, which is Ca²⁺ insensitive (25).

Phosphate Binding to F-ADP Actin Inhibits Severing by Gelsolin s1–3—Bacterially expressed gelsolin N-terminal fragment s1–3 displaced TRITC-phalloidin from F-actin in the absence of divalent cation (data not shown), in agreement with previous studies using the increase in the depolymerization of pyrene non-fluorescent labeled F-actin to assay for severing (25). Addition of gelsolin s1–3 to TRITC-phalloidin-bound F-ADP-actin displaced all of the phalloidin within 5 min (Fig. 2), a rate similar to that observed with intact human plasma gelsolin at high Ca²⁺ levels. In contrast, addition of gelsolin s1–3 to F-ADP-Pₚ-actin generated by incubation in buffer containing 50 mM Pₚ, pH 6.8, led to only a 40% loss of the enhanced fluorescence.

Gelsolin interacts with the phosphates of phosphoinositide lipids (28), and with ATP (29). However, it appears unlikely that the effect of Pₚ is on gelsolin rather than actin. Increasing concentrations of gelsolin s1–3 do not lead to an increased loss of F-actin (Fig. 2), consistent with decreased substrate availability. Also, incubation of gelsolin s1–3 in 50 mM Pₚ buffer before addition to TRITC-phalloidin-bound F-ADP-actin led to complete severing which could not be distinguished from controls (data not shown).

A previous study demonstrated a binding site for the HPO₄²⁻ ion (15) on F-ADP-actin, based on the apparently tighter binding at pH 6.8 compared with pH 8.0, where the HPO₄²⁻ ion is more prevalent. We used a similar approach to define the specific phosphate ion involved in generating gelsolin-resistant actin by determining the phosphate concentration and pH dependence of inhibition of severing by gelsolin s1–3. Consistent with the results using human plasma gelsolin, generation of F-actin resistant to severing by gelsolin s1–3 requires tens of millimolar Pₚ at pH 6.8 (Fig. 3). In contrast, at pH 8.0, 50% inhibition is detected at 12 mM Pₚ, and 25 mM maximally reduces severing. These results suggest that it is the binding of

FIG. 1. Gelsolin severs F-ADP-Pₚ actin less effectively than it does F-ADP actin. 40 µm G-ADP actin was polymerized overnight in either F-ADP buffer, pH 7.4, or F-ADP buffer containing various concentrations of Pₚ, pH 7.4, as described under "Materials and Methods." The increase in fluorescence of 1 µm TRITC-phalloidin in F-ADP and F-ADP-Pₚ-filaments was measured upon addition of 400 nM F-actin and found to be identical for both ADP and ADP-Pₚ-filaments (data not shown). Addition of 200 nM human plasma gelsolin displaced all the TRITC-phalloidin within 5 min from F-ADP actin (open circles) at all but the lowest Ca²⁺ concentration. Addition of 200 nM gelsolin to F-ADP-Pₚ-filaments (closed circles, Pₚ concentration indicated to the right of the data point) consistently displaced less of the TRITC-phalloidin than that observed for F-ADP at equivalent Ca²⁺ concentrations. Free Ca²⁺ was determined as described under "Materials and Methods."
HPO$_4^{2-}$, not H$_2$PO$_4^{2-}$, which generates the severing-resistant state. However, we cannot exclude a more complex mechanism by which the binding of both ions generates the severing-resistant state.

Binding of P$_i$ to F-ADP actin, as indicated in Fig. 3, causes a dramatic reduction in the extent of severing, when assayed over a relatively short period of time. However, even at 50 mM P$_i$, pH 8.0, there is some small amount of fluorescence loss on this timescale. To address whether the inhibition observed was actually a reduction in the kinetics of severing, we continued the analysis over a longer time scale. As illustrated in Fig. 4, gelsolin will sever ADP-P$_i$ F-actin, but requires thousands of seconds to do so. It should also be noted that the loss of fluorescence is not well described by a single exponential function.

Effects of Beryllium Fluoride and Aluminum Fluoride—BeF$_3$ and AlF$_3$ have been reported to have effects similar, but not identical, to that of P$_i$ upon binding to F-ADP actin (3, 4, 17, 20). As illustrated in Fig. 5, both BeF$_n$ and AlF$_n$ bound to F-actin reduce the ability of gelsolin to sever filaments. The concentration dependence of inhibition by BeF$_n$ is consistent with the previously reported binding data, which indicated a saturation of binding by 100 mM BeF$_n$. However, we observed an apparent reduction in the ability of BeF$_n$ to inhibit severing at concentrations higher than 100 mM, which may be related to BeF$_n$ binding to a postulated second binding site (17). This result lead us to re-examine the binding of BeF$_n$ to F-ADP actin.

Binding of BeF$_n$ to F-ADP actin reduces the fluorescence of pyrene iodoacetamide-labeled F-ADP actin (17). Incubation of 10 mM F-PIA-ADP actin with either 10 or 100 mM BeF$_n$ depresses the pyrene fluorescence with a second order rate constant of approximately 20 M$^{-1}$ s$^{-1}$ (Fig. 6A). The extent and kinetics of fluorescence depression are similar to those previously reported (17). In contrast, 1 mM BeF$_n$ caused an increase in fluorescence of F-PIA actin, not a decrease, with a rate constant of approximately 1 M$^{-1}$ s$^{-1}$. This increase in the fluorescence of F-PIA actin upon exposure to BeF$_n$ concentrations greater than 100 mM was quite apparent in an independent experiment examining equilibrium effects (Fig. 6B). These results provide further evidence for two BeF$_n$ binding sites on F-ADP actin.

Inhibition of severing by AlF$_n$ requires higher concentrations than BeF$_n$. As illustrated in Fig. 5B, 100 mM AlF$_n$ has little effect on the rate of severing, while 0.5 mM shows a significant reduction. In contrast to BeF$_n$, AlF$_n$ did not show a reduction in inhibitory activity within the concentration range tested. The concentration dependence of AlF$_n$ action is consistent with previous reports comparing the ability of AlF$_n$ and BeF$_n$ to recreate the ADP-P$_i$ like state on F-actin (17).
AlF<sub>n</sub> and BeF<sub>n</sub> do not appear to affect gelsolin directly. Incubation of gelsolin s1–3 in maximally inhibitory concentrations of AlF<sub>n</sub>, BeF<sub>n</sub>, or Pi followed by dilution into buffer lacking these molecules had no effect on the kinetics and extent of severing (data not shown). This result is not due to rapid dissociation of the ion from gelsolin. Gelsolin s1–3 severed F-ADP actin incubated for 5 min in the presence of 1 mM AlF<sub>n</sub>, with kinetics similar to that observed in the absence of AlF<sub>n</sub> (Fig. 7). In contrast, overnight incubation of F-ADP actin in 1 mM AlF<sub>n</sub> strongly reduced the susceptibility to severing by gelsolin s1–3. This result, and the fact that increasing gelsolin s1–3 concentration does not increase the amount of ADP-Pi actin severed (Fig. 1) strongly suggest that the target of these agents is the actin filament, not gelsolin.

**DISCUSSION**

A significant fraction of cellular ATP metabolism is associated with polymerization of actin (30), yet the function of actin's polymerization-stimulated ATPase is unclear. ATP hydrolysis is not necessary for polymerization, since ADP-G actin will polymerize (10). Several potential roles for ATP hydrolysis have been suggested, including regulating the structure of the actin filament (1–4, 31), regulating interactions with actin binding proteins (7, 8, 32, 33), and regulating the dynamics of monomer and filament interaction (6, 34), effects which are not necessarily mutually exclusive.

While the exact role of ATP hydrolysis in actin dynamics remains unclear, there is some agreement on the mechanistic details. Association of G-ATP actin monomer with a filament end is rapidly followed by hydrolysis of the ATP (35). However, the release of hydrolysis products is relatively slow, with ADP released upon monomer dissociation from the filament and the Pi generated from hydrolysis released at a rate approximately 2 orders of magnitude slower than ATP hydrolysis (13). Rapid polymerization or elongation of actin filaments would produce...
domains within individual filaments preferentially enriched in ATP, ADP-P_i, and ADP. Under cellular conditions ATP hydrolysis by actin is postulated to be a vectorial, not stoichiometric process (36). Therefore, domains enriched in ADP-P_i or ADP would be large and continuous, rather than dispersed throughout the filament length.

Several protein families have been proposed to regulate or enhance the disassembly of actin filaments in cells. The work described here and elsewhere suggests that members of both the gelsolin and the cofilin/destrin families of severing proteins cannot sever ADP-P_i-rich F-actin (37). Furthermore, the presence of P_i on the actin filament dramatically reduces monomer dissociation (6, 38), preventing the disassembly of actin filaments by the monomer sequestering proteins found in cells. We speculate that in cells the activation of actin filament disassembly by filament severing and monomer sequestering proteins is regulated in part by the relative proportions of ADP and ADP-P_i bound to the filament. These species, in turn, depend on the age of the actin filament and the energy charge of the cell.

The binding of F-actin of BeF_n, AlF_n, and to a lesser degree P_i increases the stability of the actin filament, raising the denaturation temperature from 65 °C to as high as 82 °C (4). Similarly, 10 mM P_i reduces the susceptibility of filaments to denaturation by SDS or KI (39). One mechanism by which the multiple effects of creating P_i-rich domains on actin filaments can be explained is by the alteration of actin filament structure (1). Binding of P_i, BeF_n, or AlF_n alters the position of subdomain 2 of the actin monomer in the actin filament (3, 19). In the ADP-P_i state this domain interacts strongly with the next monomer along the long pitch helix in the filament. In the absence of bound P_i, this domain either has multiple orientations, or is fixed in a position that interacts weakly if at all with the next monomer (3).

The structural difference between ADP-actin and ADP-P_i-actin can account for the reduced dissociation of actin monomers from the filament and the reduced ability of severing molecules to disrupt the filament. Monomer dissociation requires the coordinate disruption of multiple contacts with other monomers in the filament. Increasing the number of these contacts reduces the probability of dissociation. Furthermore, gelsolin has been suggested to sever actin filaments by interrupting the actin-actin contacts along the long-pitch helix of the filament (40). Enhancing the strength of these contacts through the increased interaction of subdomain 2 of one monomer with subdomain 1 of the next (3) should make it more difficult to sever the filament.

The binding of P_i, and BeF_n, to F-ADP actin has been characterized (3, 15–17, 20). Direct measurements demonstrate a binding site for HPO_4^{2-} with an affinity of several millimolar (15). Similarly, BeF_n (exact stoichiometry unknown) has been reported to bind F-ADP actin with a K_d of 30 μM (17). These two molecules compete with each other for binding to F-ADP actin (17). However, these results do not preclude potential lower affinity binding sites for these molecules. The binding of HPO_4^{2-} to a site on F-ADP actin is consistent with its effects on the off-rate of monomers (6, 15) and the inhibition of small molecular weight severing proteins like actophorin (37). However, the pH sensitivity and concentration dependence of severing inhibition reported here is more consistent with the binding of HPO_4^{2-} to F-ADP actin. Furthermore, the presence of two binding sites for P_i-like molecules on the actin filament is supported by the bimodal effects of BeF_n on the fluorescence of F-PIA-ADP actin.

The data presented here and in previously published work provide evidence of binding sites for both HPO_4^{2-} and HPO_4^{2-}, or their BeF_n equivalents, on the actin filament. These two sites would be sequentially filled in the release of P_i, generated by ATP hydrolysis upon actin polymerization. In such a reaction HPO_4^{2-} is released upon ATP hydrolysis, where it remains bound in the nucleotide pocket until titrated to H_2PO_4^{-}, which is slowly released from the actin filament. Consistent with both previous and current speculations, BeF_n was hypothesized to bind the P_i binding site first occupied upon nucleotide hydrolysis (18). Resistance of F-actin to severing by gelsolin correlates both with saturation of the high affinity BeF_n site and with binding to a low-affinity, at physiological pH, P_i site. In the context of actin turnover in the cell, newly polymerized actin filaments would first become sensitive to severing by gelsolin once HPO_4^{2-} is titrated to H_2PO_4^{-}, but degradation by cofilin-like molecules and by monomer sequestering proteins would require H_2PO_4^{-} dissociation.

The hypothesis that F-actin has binding sites for both HPO_4^{2-} and H_2PO_4^{-}, which fill sequentially upon ATP hydrolysis during polymerization and which modulate the association and activities of different actin binding proteins has implications for the turnover of actin filaments in cells. Newly polymerized actin filaments, rich in HPO_4^{2-} would be resistant to severing and depolymerization. Titration of HPO_4^{2-} to H_2PO_4^{-} would allow severing by gelsolin-like molecules, but this shortened filament would be relatively resistant to disassembly by cofilin-like molecules and to depolymerization. This gelsolin-capped filament would then have some time, based on the dissociation rate of H_2PO_4^{-}, to move to a new site in the cell, where it could uncap or incorporate into new cytoskeletal structures. If the filament is not stabilized, either by further elongation with accompanying ATP hydrolysis, or by association of other stabilizing factors such as tropomyosin, the time-dependent loss of H_2PO_4^{-} increases the probability of further fragmentation by cofilin-like molecules and depolymerization by monomer dissociation.

This model adds a temporal component to the many previous models that considered actin turnover in the context of the spatial organization of the cell. Several studies of actin turnover in cells suggest that actin filament depolymerization is not a spatially uniform process (41, 42), but the mechanism for locally altering depolymerization is undefined. Consideration of the effects of nucleotide hydrolysis by actin on filament severing and depolymerization suggests that the dynamics of...

Fig. 7. Incubation of F-ADP actin in 1 mM AlF_n does not rapidly lead to the generation of severing resistant F-actin. The severing of F-ADP actin in two control experiments (open symbols) is compared with that of G-ADP actin polymerized overnight in 1 mM AlF_n and assayed in 1 mM AlF_n (closed triangles) and with that of F-ADP actin incubated in 1 mM AlF_n for 5 min and then assayed in 1 mM AlF_n (closed squares). The F-actin and gelsolin s1-3 concentrations were 300 nM each. Time is in seconds.
filament turnover in cells are defined by the ATPase rate of actin and the titration and release of Pi, as well as the regulated action of actin binding proteins.

Acknowledgments—We thank J. Ka¨s, W. Witke, T. P. Stossel, and other members of the Division of Experimental Medicine for useful comments and suggestions.

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doi: 10.1074/jbc.271.9.4665

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