Biochemistry

Article

Cite This: Biochemistry 2019, 58, 40−47

pubs.acs.org/biochemistry

Quantitative Variations with pH of Actin Depolymerizing Factor/Cofilin's Multiple Actions on Actin Filaments

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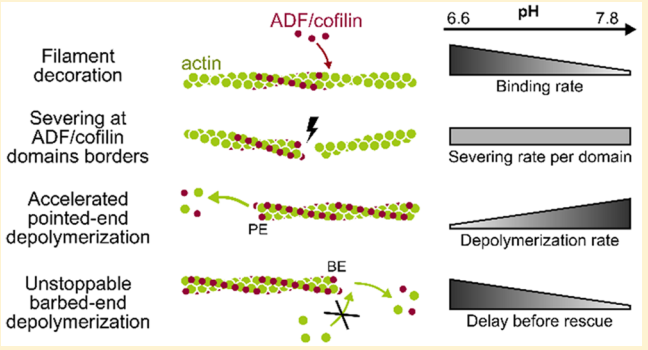
Supporting Information

ABSTRACT: Actin depolymerizing factor (ADF)/cofilin is the main protein family promoting the disassembly of actin filaments, which is essential for numerous cellular functions. ADF/cofilin proteins disassemble actin filaments through different reactions, as they bind to their sides, sever them, and promote the depolymerization of the resulting ADF/cofilin-saturated filaments. Moreover, the efficiency of ADF/cofilin is known to be very sensitive to pH. ADF/cofilin thus illustrates two challenges in actin biochemistry: separating the different regulatory actions of a single protein and characterizing them as a function of specific biochemical conditions. Here, we investigate the different reactions of ADF/cofilin on actin filaments, at four different pH values ranging from 6.6 to 7.8, using single-filament microfluidics techniques. We show that decreasing the pH decreases the effective filament severing rate by increasing the rate at which filaments become saturated by ADF/cofilin, thereby reducing the number of ADF/cofilin domain boundaries, where severing can occur. The severing rate per domain boundary, however, remains unchanged at different pH values. The ADF/cofilin-decorated filaments ("cofilactin" filaments) depolymerize from both ends. We show here that, at physiological pH (7.0−7.4), the pointed end depolymerization of cofilactin filaments is barely faster than that of bare filaments. In contrast, cofilactin barbed ends undergo an "unstoppable" depolymerization (depolymerizing for minutes despite the presence of free actin monomers and capping protein in solution), throughout our pH range. We thus show that, at physiological pH, the main contribution of ADF/cofilin to filament depolymerization is at the barbed end.



A number of key cellular processes rely on the proper assembly and disassembly of actin filament networks.[1] The central regulator of actin disassembly is the ADF/cofilin protein family,[2,3] which comprises three isoforms in mammals: cofilin-1 (cof1, found in nearly all cell types), cofilin-2 (cof2, found primarily in muscles), and actin depolymerization factor (ADF, found mostly in neurons and epithelial cells). We refer to them collectively as "ADF/cofilin".

Over the years, the combined efforts of several laboratories have led to the following understanding of actin filament disassembly by ADF/cofilin. Molecules of ADF/cofilin bind stoichiometrically[4,5] to the sides of actin filaments, with a strong preference for ADP-actin subunits.[6−10] Though ADF/cofilin molecules do not contact each other,[11] they bind in a cooperative manner, leading to the formation of ADF/cofilin domains on the filaments.[5,7,9,12,13] Compared to bare F-actin, the filament portions decorated with ADF/cofilin (termed "cofilactin") are more flexible[14,15] and exhibit a shorter right-handed helical pitch, with a different subunit conformation.[11,16−19] Thermal fluctuations are then enough to sever actin filaments at (or near) domain boundaries.[8,9,13,20,21] Cofilactin filaments do not sever but depolymerize from both ends,[13] thereby renewing the actin monomer pool.

ADF/cofilin thus disassembles actin filaments through the combination of different actions. As such, it vividly illustrates a current challenge in actin biochemistry: identifying and quantifying the multiple reactions involving a single protein. This is a very difficult task for bulk solution assays, where a large number of reactions take place simultaneously, and single-filament techniques have played a key role in deciphering ADF/cofilin's actions.[9,13,20,22−24] In particular, the microfluidics-based method that we have developed over the past years is a powerful tool for such investigations.[25] It has recently allowed us to quantify the kinetics of the aforementioned reactions and to discover that ADF/cofilin-saturated filament (cofilactin) barbed ends can hardly stop depolymerizing, even when ATP-G-actin and capping protein are present in solution.[13]

In addition, ADF/cofilin is very sensitive to pH.[4,5,26−31] In cells, the pH can be a key regulatory factor.[32] It can vary between compartments, vary between cell types, and be specifically modulated. We can consider that a typical cytoplasmic pH would be between 7.0 and 7.4. Recently, we have quantified the different reactions involving ADF/cofilin at

Special Issue: Future of Biochemistry: The International Issue

Received: September 19, 2018
Revised: November 23, 2018
Published: November 30, 2018

DOI: 10.1021/acs.biochem.8b01001
Biochemistry 2019, 58, 40−47

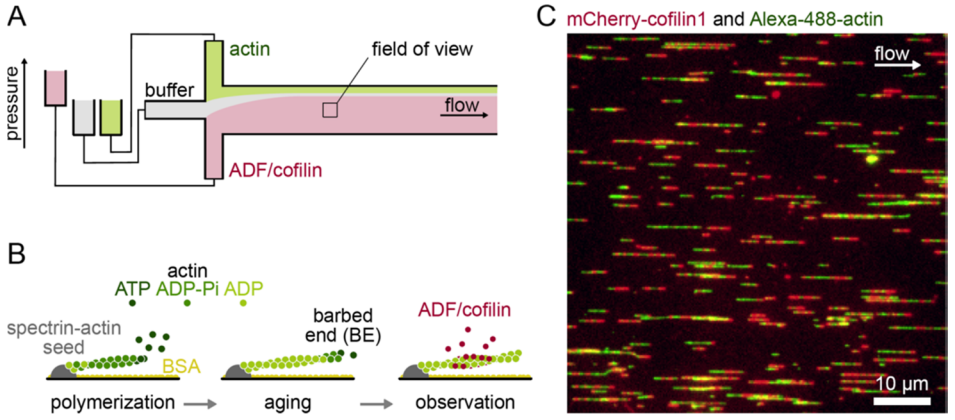


Figure 1. Using microfluidics to monitor individual actin filaments and the binding of cofilin. (A) Experiments are performed in microfluidics chambers, sketched from above. The main channel is connected through three inlets to different protein solutions. Controlling the pressure in each inlet allows one to rapidly change the solution in the field of view. (B) Sketch of a typical experiment (side view). Filaments are elongated from coverslip-anchored spectrin-actin seeds, by injecting ATP-G-actin. Filaments are then aged by flowing in a solution of ATP-G-actin at the critical concentration, for at least 15 min. This results in >99% of the monomers being in the ADP state. Finally, filaments are exposed to ADF/cofilin. (C) Example of a field of view, imaged with TIRFm. ADP-F-actin labeled with Alexa-488 is exposed to mCherry-cofilin-1, which forms observable domains on the filaments.

pH 7.8,[13] leaving open the question of how these reaction rates are individually affected by pH variations. For instance, it has been reported that ADF/cofilin is a more potent filament disassembler at higher pH values,[4,5,26−29] but the actual impact of pH on the rate constants of individual reactions has yet to be determined. Moreover, whether the unstoppable barbed end depolymerization that we have recently discovered for ADF/cofilin-saturated filaments at pH 7.8[13] remains significant at lower, more physiological pH values is an open question.

Here, we investigate how the different contributions of ADF/cofilin (using unlabeled ADF, unlabeled cof1, and eGFP-cof1) to actin filament disassembly depend on pH, which we varied from 6.6 to 7.8. We first present the microfluidics methods that we have used for the observation of individual filaments (Figure 1). We measured cofilin's ability to decorate actin filaments by binding to their sides (Figure 2) and the rate at which individual cofilin domains severed actin filaments (Figure 3). We next quantified the kinetic parameters of filament ends, for bare and ADF/cofilin-saturated (cofilactin) filaments (Figure 4), and we specifically quantified the extent to which the barbed ends of cofilactin filaments are in a state that can hardly stop depolymerizing (Figure 5). We finally summarize our results (Figure 6).

METHODS

Buffers and Proteins. Experiments were performed at room temperature in F-buffer (10 mM Hepes or Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM ATP, 10 mM DTT, and 1 mM DABCO) at different pH values: pH 6.6 (Hepes), pH 7.0 (Hepes or Tris), pH 7.4 (Tris), and pH 7.8 (Tris). The pH of each buffer was adjusted after all of the ingredients had been mixed.

All the protocols for protein purification can be found in ref 13. Actin was purified from acetone powder, made from rabbit muscle. Recombinant mouse cofilin-1, eGFP-cof1 (with eGFP at the N-terminus), human ADF, human profilin-1, and human gelsolin were expressed in bacteria and purified. Capping protein was made from recombinant mouse capping proteins alpha1 and beta2, co-expressed in bacteria. Spectrin-actin seeds were purified from human erythrocytes.

Gelsolin was biotinylated with sulfo-NHS-biotin. Actin was fluorescently labeled on accessible surface lysines of F-actin, using Alexa488 or Alexa568 succinimidyl ester.

Microfluidics for the Study of Single Actin Filaments. To distinguish the different actions of cofilin and quantify them, one needs to observe single events on individual actin filaments. To do so, the microfluidics-based method that we have developed over the past 9 years[25] is a valuable tool. The microfluidics setup is sketched in Figure 1A. It allows one to monitor a large number of actin filaments anchored by one end only, as well as labeled cofilin, using epifluorescence or TIRF microscopy (Figure 1B). The experiments we report here are very similar to the ones we described in ref 13. Barbed end dynamics, as well as cofilin side binding and filament severing, were monitored on filaments grown from spectrin-actin seeds anchored to the coverslip surface (Figure 1B). Pointed end dynamics were monitored by anchoring gelsolin-capped barbed ends to the coverslip surface, using biotinylated gelsolin and a neutravidin-decorated surface.

Microscopy and Data Analysis. Images were acquired via epifluorescence or TIRF microscopy (ILAS2, Roper Scientific, now Gataca systems) on a Nikon TiE inverted microscope equipped with a 60× oil-immersion objective, either with an Evolve EMCCD camera (Photometrics) controlled by Metamorph or with an Orca-Flash2.8 camera (Hamamatsu) controlled by Micromanager. Images were analyzed using ImageJ.

Elongation or depolymerization rates (Figure 4) were determined on individual filaments, and median values were reported. We considered that each actin subunit contributed 2.7 nm to the filament length. For the quantification of severing (Figure 3), uncapping (Figure 5C), and rescue (Figure 5E,F), survival fractions were determined, following a Kaplan−Meier algorithm.[33]

As an example, we detail here the protocol for the quantification of severing (Figure 3). Filaments were polymerized from anchored spectrin-actin seeds with Alexa-568 (14%)-actin and aged for at least 15 min to ensure that >99% of the monomers were in an ADP state.[25] A solution of low-concentration eGFP-cofilin-1 in F-buffer (no G-actin) was then constantly injected. Images were acquired using

DOI: 10.1021/acs.biochem.8b01001
Biochemistry 2019, 58, 40−47

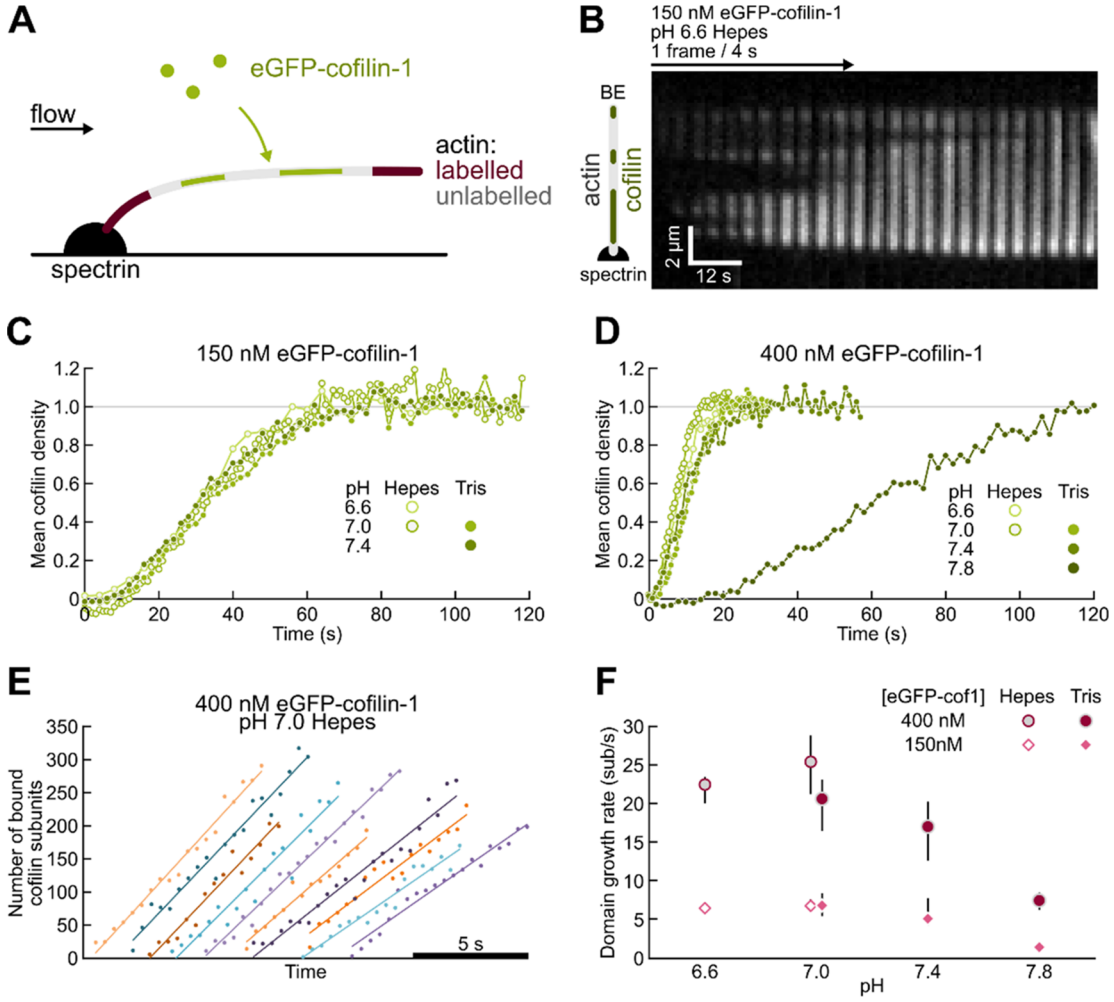


Figure 2. Cofilin binds more slowly to filaments at higher pH values. (A) Experimental configuration. Actin filaments are grown from spectrin-actin seeds with a long middle segment of unlabeled ADP-actin. (B) Time-lapse image showing an unlabeled ADP-actin filament becoming saturated by eGFP-cof1 over time. (C and D) Mean normalized eGFP-cofilin-1 fluorescence signal, binding to unlabeled ADP-F-actin. eGFP-cofilin-1 at concentrations of (C) 150 and (D) 400 nM was injected in the chamber from time zero onward. The fluorescence signal was averaged along 20−35 pixels (3.5−6 μm) for each filament. There were (C) N = 10, 10, 18, and 20 filaments used for pH 6.6 Hepes, pH 7.0 Hepes, pH 7.0 Tris, and pH 7.4 Tris, respectively, and (D) N = 10 filaments for each condition. (E) Number of cofilin subunits in individual domains, increasing over time. For the sake of clarity, the time origin has been shifted for each curve. Lines show the linear fit. Condition: 400 nM eGFP-cofilin-1 and pH 7.0 Hepes. (F) Growth rates of individual cofilin domains at different eGFP-cofilin-1 concentrations and pH. Values are median values; error bars show the interquartile range. N = 10 domains, except N = 9 for pH 6.6 Hepes with 150 nM cof1 and for pH 7.8 Tris with 400 nM cof1.

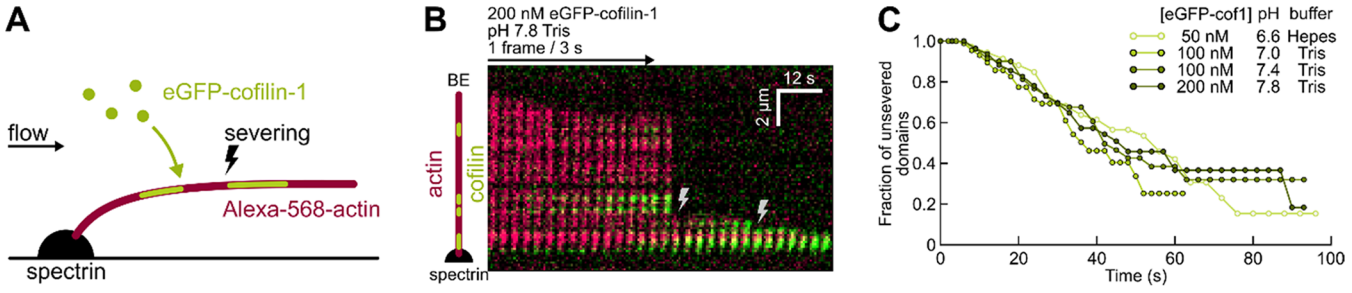


Figure 3. The severing rate per cofilin domain is unaffected by pH. (A) Experimental setup. Alexa-568-labeled actin filaments are polymerized from actin-spectrin seeds and aged before being exposed to eGFP-cof1. (B) Typical kymograph. eGFP-cof1 at a concentration of 200 nM (green) is constantly injected, binds F-actin (red), and induces severing (lightning symbols). (C) Fraction of cofilin domains with no severing event detected near their edges over time. Time zero is defined for each domain as the last frame before the domain becomes visible. The survival fraction curves are calculated using the Kaplan−Meier method over 22−43 filaments, 78−90 cofilin domains, and 30−33 severing events for each data set.

epifluorescence microscopy. All domains located at least 0.5 μm (4 pixels) from the anchored seed were analyzed. For each domain, time zero was defined as the frame before which they appeared. Domains could then "sever", i.e., have a filament severing event occur near one of their boundaries, or be "lost", for example when a severing event occurred at another domain

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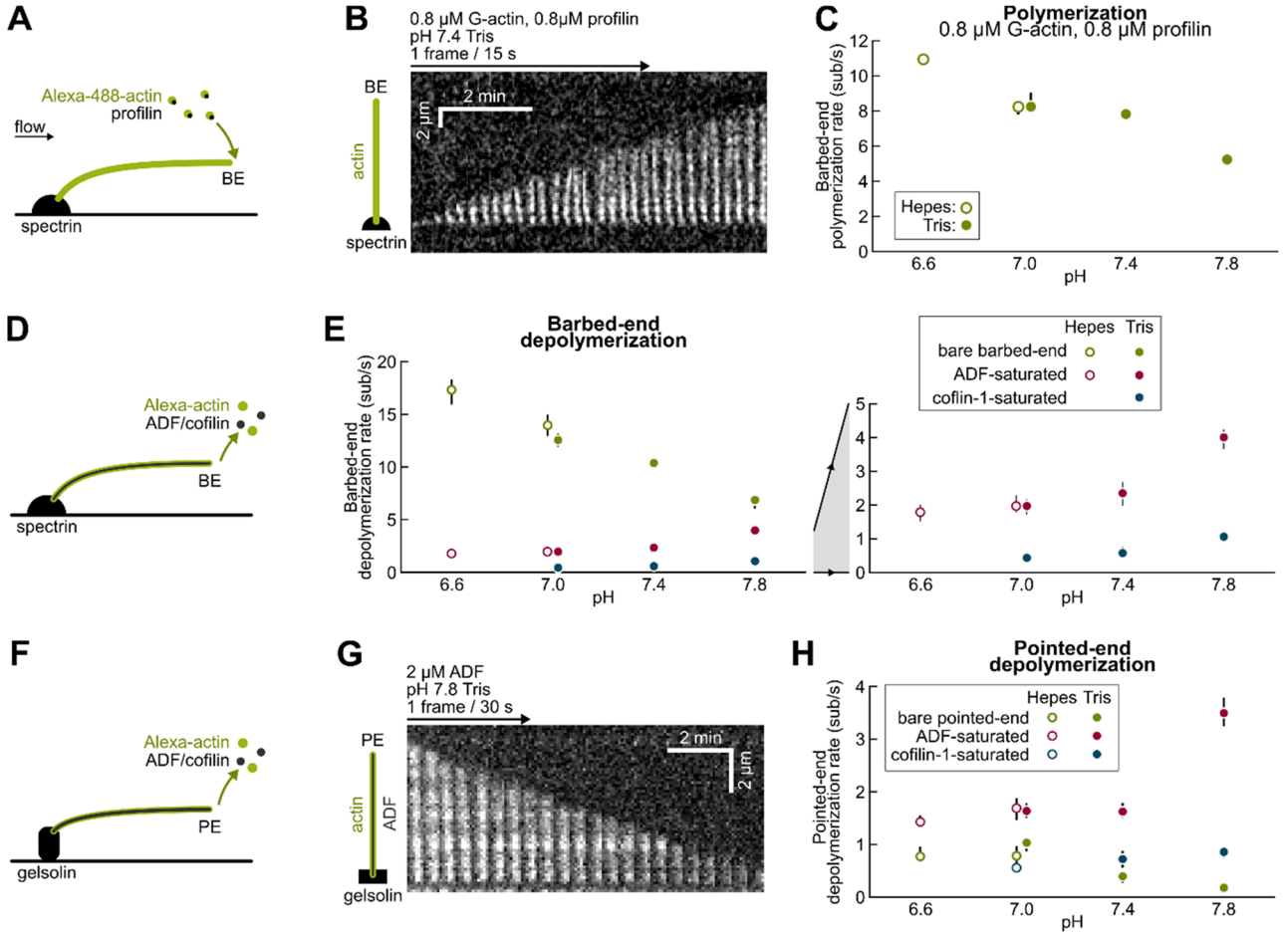

Figure 4. A higher pH slows polymerization and depolymerization of bare F-actin but accelerates depolymerization of ADF/cofilin-saturated filaments at both ends. (A–C) Polymerization from the barbed end. (A) Sketch of the experimental configuration, where filaments were grown from actin-spectrin seeds with G-ATP-actin and profilin. (B) Kymograph of a typical elongating filament. (C) Polymerization rate at different pH values. $N = 20$ filaments for each condition. (D and E) Depolymerization from the barbed end. (D) Sketch of the experimental configuration. ADP-F-actin is exposed either to buffer only or to 1–2 μM unlabeled ADF or cofilin-1 to fully saturate the filament in <1 min. (E) Depolymerization rate for different pH values. The right panel shows a close-up of the 0–5 sub/s range. From left to right, $N = 20$, 32, 22, 32, and 31 (bare filaments), $N = 9$, 14, 23, 33, and 34 (ADF-saturated), and $N = 17$, 18, and 16 (cofilin-1-saturated). (F–H) Depolymerization from the pointed end. (F) Sketch of the experimental configuration. ADP-F-actin was bound to the surface by gelsolin. Filaments were exposed to buffer only (supplemented with 0.4 mM $CaCl_2$ to ensure gelsolin-actin tight binding) containing 1–2 μM unlabeled ADF or cofilin-1 to rapidly saturate filaments. (G) Typical kymograph of a depolymerizing filament saturated with ADF. (H) Pointed end depolymerization rate at different pH values. $N = 14$, 20, 15, 20, and 20 (bare filaments). $N = 20$, 20, 16, 20, and 20 (ADF-saturated). $N = 20$, 20, and 20 (cofilin-1-saturated). In panels C, E, and H, symbols show median values and error bars show the interquartile range.

located upstream on the same filament. These events were accounted for using a Kaplan–Meier algorithm to determine the survival fraction of unsevered domains over time (Figure 3C).

RESULTS AND DISCUSSION

Cofilin Binds Faster to Actin Filaments at Lower pH Values. Using our microfluidics setup, we have generated ADP-actin filaments comprising a long unlabeled segment and have monitored the binding of eGFP-cof1 to this segment (Figure 2A,B). We found that the decoration of the filament was equally fast at pH 6.6, 7.0, and 7.4 (Figure 2C) but significantly slower at pH 7.8, where it took approximately 6 times longer to reach 50% of full saturation in the presence of 400 nM eGFP-cof1 (Figure 2D).

We measured the growth rate of individual eGFP-cof1 domains (Figure 2E) and found that they appeared to grow symmetrically toward both filament ends, as we already reported for pH 7.8.[13] As we found in our observation for the overall decoration of filaments, we found that domain growth rate was pH-independent for low pH values and approximately 3-fold lower at pH 7.8 (Figure 2F). Overall, our results show that cofilin domains nucleate and grow much faster at pH 6.6–7.4 than at pH 7.8.

The Severing Rate per Cofilin Domain Is Unaffected by pH. We next sought to measure the severing rate per eGFP-cof1 domain at different pH values. To do so, we exposed Alexa-568 (14%)-ADP-actin filaments to eGFP-cof1 and monitored the severing events over time for each cof1 domain (Figure 3). As previously reported, severing events were observed to occur at the boundaries of cof1 domains and occurred more often at the pointed end side of the domain. Different cofilin concentrations were used at different pH values to observe separate, individual domains long enough (domains grow faster and thus fuse more rapidly at lower pH

DOI: 10.1021/acs.biochem.8b01001
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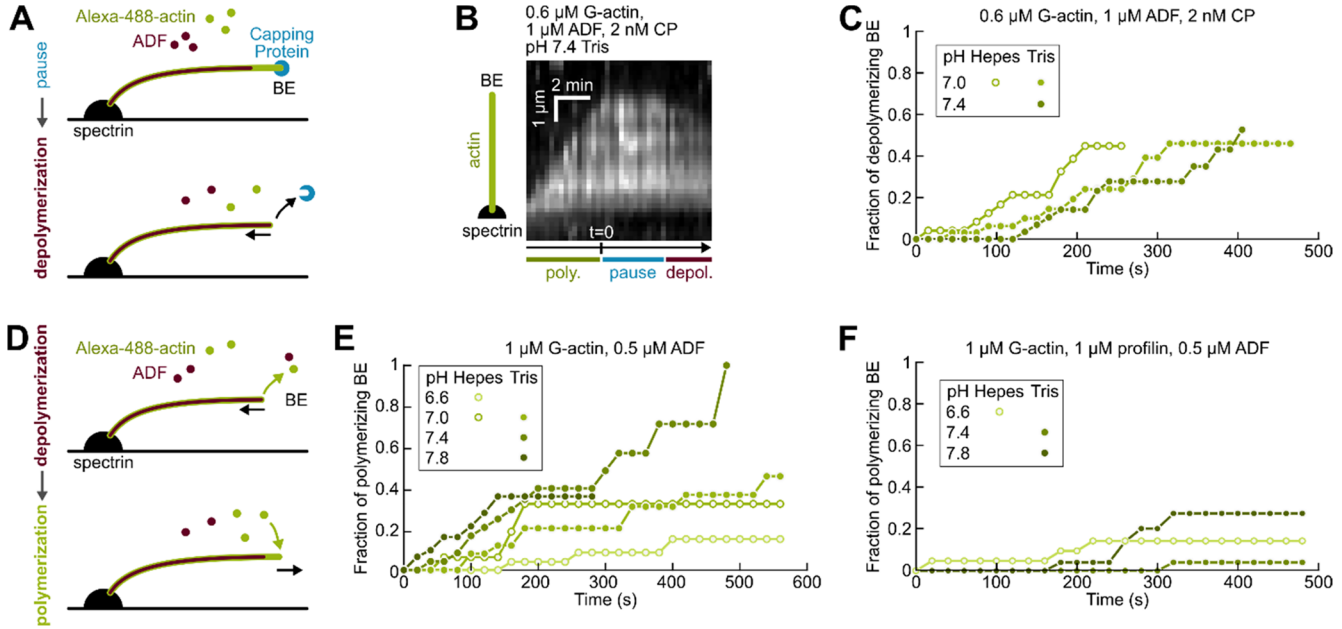

Figure 5. "Unstoppable" depolymerization of cofilactin barbed ends is observed throughout our pH range. (A–C) Synergy of CP and ADF/cofilin in saturating filaments and initiating barbed end depolymerization. (A) Sketch of the experimental configuration and events. Filaments grow until they are capped with CP. ADF/cofilin can then saturate the filaments, up to their BE, which thus uncaps and depolymerizes. (B) Kymograph of a filament continuously exposed to the same solution containing 0.8 μM G-ATP-actin, 1 μM ADF, and 2 nM CP. The filament polymerizes, pauses as it is capped by CP, and eventually depolymerizes. (C) Fraction of barbed ends that underwent a transition from a pause to depolymerization. Time zero corresponds to the beginning of the pause (as shown in panel B). N = 24, 32, and 32 filaments for pH 7.0 Hepes, pH 7.0 Tris, and pH 7.4 Tris, respectively. (D–F) Cofilactin barbed ends sustain depolymerization in the presence of ATP-G-actin. (D) Sketch of the experimental configuration and events. Filaments are polymerized from spectrin-actin seeds and saturated with ADF. Depolymerizing cofilactin filaments are then constantly exposed to a solution of ATP-G-actin. (E) Fraction of barbed ends that underwent a transition from depolymerization to polymerization over time when exposed to 1 μM ATP-G-actin and 0.5 μM ADF (to keep filaments saturated). N = 25, 16, 25, 24, and 31 for pH 6.6 Hepes, pH 7.0 Hepes, pH 7.0 Tris, pH 7.4 Tris, and pH 7.8 Tris, respectively. (F) Same as panel E but with 1 μM profilin added to the solution. N = 21, 27, and 30 for pH 6.6 Hepes, pH 7.4 Tris, and pH 7.8 Tris, respectively.

values). We found no significant differences in the severing rate per domain as a function of pH (Figure 3C).

Therefore, our results indicate that the previously reported enhancement of filament severing activity by cofilin at higher pH values[34] is not due to faster severing at each potential severing site but to a greater number of these sites, i.e., a greater number of domain boundaries. For a given concentration range, the rapid cofilin decoration at low pH makes domain boundaries shorter-lived, as domains rapidly expand and merge.

Bare Actin Filaments (without Cofilin) Are More Dynamic at Lower pH Values. Before measuring the depolymerization rates of ADF/cofilin-saturated filaments, we measured the barbed end elongation rate as well as the depolymerization rate of both ends in the absence of ADF/cofilin at different pH values (Figure 4). We found that barbed ends exhibited higher on and off-rates at low pH (Figure 4C,E) and that pointed ends also had higher off-rates at low pH (Figure 4H). This is consistent with earlier work on pH,[35−37] and studies performed at high pH values[25] typically report filament dynamics slower than those from studies performed at lower pH values.[38]

ADF/Cofilin-Saturated Filaments Depolymerize Faster at Higher pH Values. The pointed end depolymerization of ADF- and cof1-saturated filaments is faster at higher pH values (Figure 4H). As a result, the enhancement of pointed end depolymerization by ADF saturation, which is very significant at pH 7.8 (a 17-fold increase, compared to that of bare filaments), is milder at physiological pH (a 4-fold increase at pH 7.4 and a 2-fold increase at pH 7). cof1-saturated filament pointed ends at physiological pH (7.0−7.4) depolymerize at rates similar to those of bare filaments. This effect likely contributes to the more efficient filament disassembly previously reported for higher pH values[4,5,26−29] (in addition to severing, which we have discussed above).

When filaments were saturated with cof1 or ADF, barbed ends depolymerized more slowly than those of bare filaments, and their off-rate increased with pH (Figure 4E). Consequently, the difference in barbed end depolymerization between bare and saturated filaments was greater at lower pH values: ADF-saturated barbed ends depolymerized 6.6-fold slower than bare barbed ends at pH 7 but only 1.7-fold slower at pH 7.8.

Another totally different effect of ADF/cofilin on barbed end dynamics is that free ADF/cofilin molecules in solution directly target bare ADP-actin barbed ends and increase the monomer off-rate, as we have first reported at pH 7.8.[13] This effect remains at lower pH (Figure S1). This effect, which requires ADP-actin at the barbed end, is unlikely to play a role in cells, where an ATP-actin monomer will quickly bind the barbed end and thus protect it from the direct targeting by ADF/cofilin.[13] Moreover, this enhancement of depolymerization by direct targeting of the barbed end disappears if the sides of the filament are decorated with ADF/cofilin up to the barbed end. At physiological pH (7.0−7.4), the faster decoration of the filament sides by ADF/cofilin (Figure 2) makes this direct targeting of the BE even less likely to play a role in cells.

Nonetheless, the effect of direct BE targeting by ADF/cofilin can be readily observed in vitro, if actin monomers are absent

DOI: 10.1021/acs.biochem.8b01001
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from solution and the barbed end thus remains ADP-actin (Figure S1). Importantly, this effect should not be confused with the saturation of the sides of the filaments with ADF/cofilin, which slows barbed end depolymerization (Figure 4E). This was unfortunately the case in a recent study[39] in which the authors, using unlabeled ADF, wrongly concluded that binding ADF to the sides of filaments accelerated their depolymerization from the barbed end.

Depolymerization at the Barbed Ends of Cofilactin Filaments Is Even Harder To Stop at Lower pH Values. We next investigated if the unstoppable barbed end depolymerization of ADF/cofilin-saturated filaments, which we discovered at pH 7.8,[13] also occurred at lower pH values.

We verified that, at physiological pH (7.0−7.4), capped filaments exposed to ADF became uncapped and started to depolymerize (Figure 5A−C). Because lower pH values accelerate the formation and growth of cofilin domains on filaments [observed for cof1 (Figure 2)], they also decrease the time required for these domains to reach the barbed end and uncap it: at pH 7.0 and 7.4 (Figure 5C), uncapping occurs faster than what we previously observed at pH 7.8.[13] To further quantify the unstoppable nature of barbed end depolymerization for ADF/cofilin-saturated filaments, we compared the time it took for 1 μM ATP-G-actin to "rescue" ADF-saturated filament barbed ends from depolymerization. We found that this rescue was slower at lower pH values (Figure 5E). We found that adding profilin in the buffer delayed further the rescue of depolymerizing barbed ends (Figure 5F).

Our results thus show that the "unstoppable" depolymerizing state of cofilactin barbed ends is a feature that exists over the whole pH range that we have explored. In fact, its contribution to the depolymerization of cofilactin filaments appears to be greater at physiological pH (7.0−7.4) than at pH 7.8 where it was originally discovered[13] for the following reasons. (1) Depolymerizing cofilactin barbed ends are more difficult to rescue at lower pH values. (2) Capped actin filaments are more rapidly saturated by ADF/cofilin and uncapped at lower pH values. (3) ADF-saturated filaments depolymerize faster from their barbed ends than from their pointed ends, which depolymerize almost as slow as the pointed ends of bare actin filaments at lower pH values.

CONCLUSIONS

Our results further illustrate the power of single-filament microfluidics as a tool for actin biochemistry. We could not have obtained these results with bulk solution assays, and microfluidics offered a number of advantages compared to standard single-filament techniques.[25,40,41] Here, it allowed us to distinguish and to separately quantify the main reactions of ADF/cofilin on actin filaments for different pH values: binding to the sides (Figure 2) and severing (Figure 3) of actin filaments and, for filaments decorated by ADF/cofilin, the acceleration of pointed end depolymerization (Figure 4) and the unstoppable depolymerization of barbed ends (Figure 5). The quantitative variations of these reactions with pH are summarized in Figure 6.

Overall, our results are consistent with the notion that decreasing the pH mostly affects the conformation of the actin filament, which is then more favorable for binding ADF/cofilin.[42] Indeed, at lower pH values, we find that bare actin filaments are less cohesive and depolymerize faster and that decoration by ADF/cofilin makes them more stable because of the additional bonds it provides. Our observation that ADF/cofilin binds more readily to actin filaments at lower pH values is also consistent with the idea that actin filaments are in a more "cofilin-friendly" conformation. These changes in F-actin conformation with pH likely involve the binding of cations to specific sites on the subunits, which modulate filament properties[43] and whose reorganization is coupled to cofilin binding.[44]

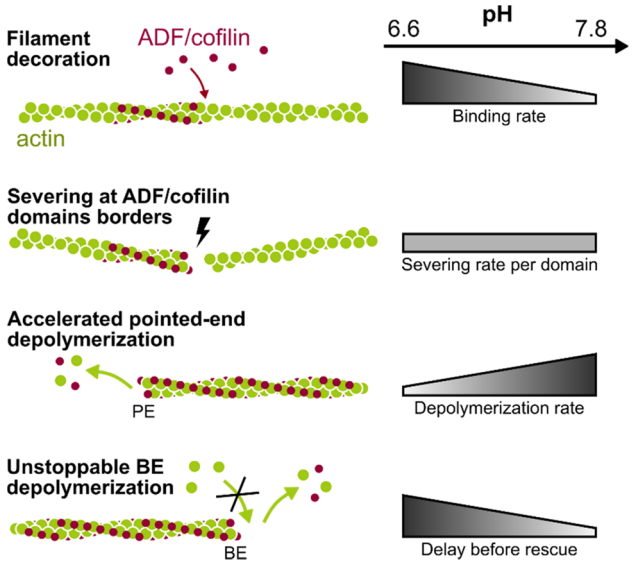

Figure 6. Summary of results. Barbed end depolymerization is an important contribution of cofilin disassembly at physiological pH. Within the pH range that we have explored (6.6−7.8), we have made the following observations (from top to bottom, on this sketch). A lower pH favors the rapid decoration of filaments by ADF/cofilin, but the severing rate per cofilin domain does not vary with pH. As a consequence, at a higher pH, domain boundaries persist longer (before domains merge) and severing is more efficient. The acceleration of pointed end depolymerization for cofilactin filaments is mostly observed at high pH values. The "unstoppable" depolymerization of cofilactin barbed ends is observed at all pH values and is more pronounced at lower pH values.

Faster ADF/cofilin binding at lower pH values also explains why previous studies have reported a weaker severing activity at lower pH values: domain boundaries, where severing can occur, rapidly vanish as domains rapidly expand and merge. We found that the severing rate per domain was unaffected by pH, within the range we explored. Importantly, we found that cofilactin filament pointed ends did not depolymerize much faster than bare filament barbed ends at physiological pH values. In contrast, the unstoppable barbed end depolymerization of cofilin-saturated filaments remains an important feature at all pH values and is even stronger for lower pH values. Our results thus show that, at physiological pH, the dominant effect differentiating cofilactin filaments from bare filaments is the nature of their barbed ends and their "unstoppable" depolymerization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b01001.

Acceleration of barbed end depolymerization by ADF/cofilin in solution (Figure S1) (PDF)

DOI: 10.1021/acs.biochem.8b01001
Biochemistry 2019, 58, 40−47

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Funding

The authors acknowledge funding from the Human Frontier Science Program (Grant RGY0066 to G.R.-L.), French Agence Nationale de la Recherche (Grant Muscactin to G.R.-L.), the European Research Council (Grant StG-679116 to A.J.), and Fondation ARC pour la Recherche sur le Cancer (postdoctoral fellowship to H.W.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank all members of the Romet-Lemonne/Jegou lab and especially Bérengère Guichard and Sandy Jouet for help with buffers and protein purification as well as Emiko Suzuki for her careful reading of the manuscript.

ABBREVIATIONS

ADF, actin depolymerizing factor; BE, barbed end; PE, pointed end; F-actin, filamentous actin; G-actin, globular (monomeric) actin; cofilactin, cofilin (or ADF)-decorated actin filament; TIRFm, total internal reflection fluorescence microscopy.

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DOI: 10.1021/acs.biochem.8b01001
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