Kinetic overshoot in actin network assembly induced jointly by branching and capping proteins

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We report an experimental study of the kinetics of actin assembly mediated by branching and capping proteins. Our findings confirm the recent prediction of a "branching explosion" occurring during polymerization. Fluorescence imaging shows a number of actin filaments with branches within a few minutes of polymerization, induced by the activated branching protein complex Arp2/3, but the number of visible branches decreases over time. The light-scattering intensity displays an overshoot as a function of time, which we attribute to the formation of highly branched clusters early in polymerization. Furthermore, the overshoot occurs over a limited range of the ratio of concentrations of branching and capping proteins, also consistent with the theoretical model. These results establish a natural link between the kinetic theory of actin assembly *in vitro* and the cytoskeletal structure and actin dynamics in motile cells.

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I. INTRODUCTION

Most eukaryotic cells utilize the intracellular protein actin as the primary component for shape regulation, force generation, and migration [1,2]. At the core of these functions is the regulated assembly of actin into filaments and subsequently more complex networks involving an array of accessory proteins [3,4]. Whereas many actin-based functions also involve its counterpart motor protein myosin, recent studies have shown that actin-based motility can be produced by an essential set of purified proteins not including myosin [5–7]. As illustrated in Fig. 1, this set of proteins include the actin-related-protein Arp2/3 complex as the branching agent [8] and a capping protein such as Cap-Z or gelsolin [5,9]. Arp2/3 complex (MW=224 kDa) is a seven-unit protein assembly that, when activated by agents such as the bacterial surface protein ActA [10] or its mammalian analog, the Wiskott-Aldrich syndrome protein (WASP) [11], initiates branching and polymerization of actin, leading to the formation of a dendritic network. This complex preferentially binds to the side of a pre-existing "mother" filament, forming a nucleation site for a "daughter" branch of newly polymerized F-actin that extends from the mother filament at an angle of ~70° [12]. This polymerization process is autocatalytic because the growth of the daughter filaments is enhanced by the presence of mother filaments [13]. A capping protein such as Cap-Z or gelsolin caps the plus (growing) end of F-actin [9], thereby regulating growth and preserving a supply of free monomers. Addition of the capping protein is also shown to alter the density and structure of the composite protein network, thereby enhancing the growth of the actin comet tails reconstituted *in vitro* [7].

Along with extensive experimental studies of branched actin structures both *in vitro* and *in vivo*, theoretical efforts have investigated the dependence of the structure and polymerization kinetics on key rates such as those of branching and capping. Recently, one of us (A.E.C.) calculated the average branching number and cluster size in the autocatalytically branched actin solutions containing activated Arp2/3 complex and a capping protein, using analytic theory and computer simulations [13]. It was found that the average number of daughter branches (n_{br}) growing from the side of a mother filament is less than unity at steady state: $n_{br} = \tau_{dis}/(\tau_{dis}+\tau_{depol}) < 1$, where τ_{dis} is the filament dissociation (debranching) time constant, and τ_{depol} is the depolymerization time constant. Early in polymerization, however, the kinetic analysis predicts an explosion of transient branches under appropriate conditions. This model suggests that highly branched structures are developed early in polymerization, followed by a reduction in branching over time. Simulation results for the root-mean-square number of filaments per cluster as a function of time showed a peak within minutes after the initiation of the polymerization, which should be detectable by light-scattering measurement. This prediction has not yet been confirmed.

In this article, we describe experiments that test the theoretical predictions above, using fluorescence imaging and static light-scattering techniques. We indeed observed the predicted overshoot behavior in actin polymerization, thus confirming the key prediction made both analytically and by

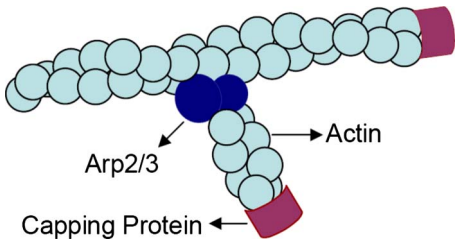

FIG. 1. (Color online) A schematic drawing of F-actin with a single branch, induced by Arp2/3 complex.

simulations [13]. Moreover, we find that the ratio between Arp2/3 complex and the capping protein gelsolin defines a limited range for the overshoot to occur, again consistent with the theoretical analysis [13].

II. MATERIALS AND METHODS

A. Sample preparation

Actin was purified from rabbit skeletal muscle as described previously [14]. The purified actin monomers were equilibrated in 10-mM Tris at pH 7.5, 0.2-mM ATP, 0.5-mM DTT, and 0.2-mM $CaCl_2$. The Arp2/3 complex and the verprolin homology cofilin homology and acidic region of WASP, noted in the literature as VCA, were both purchased from Cytoskeleton, Inc. (Denver, Colorado). Recombinant human plasma gelsolin used as the capping protein for this study was a generous gift from P. Janmey, University of Pennsylvania Medical School. Stock solutions of Arp2/3, VCA, and gelsolin were prepared in concentrations of over 10 μM so that much smaller volumes of these proteins in their recommended buffers were required to add into a solution of unpolymerized actin. Therefore, the ionic balance or pH of the actin solution was not altered when calculated amounts of these additional protein solutions were added prior to initializing actin assembly. Finally, actin polymerization was triggered by the addition of $MgCl_2$ and KCl to the final concentrations of 2 and 50 mM, respectively.

B. Fluorescence microscopy

Actin filaments were labeled by adding tetramethylrhodamine B isothiocyanate conjugated to phalloidin (TRITC-phalloidin), purchased from Sigma, Inc. (St. Louis, Missouri). This imaging technique is well-established and has been adopted in our previous work [14,15]. At selected time points into the polymerization, an aliquot of 4 μl of the polymerizing actin solution of 2 μM concentration was mixed with 1:1 molar ratio TRITC-phalloidin for one minute and diluted by 40 times in volume with the actin buffer containing all the chemical components listed above, including 2-mM $MgCl_2$ and 50-mM KCl. A 1.5 μl aliquot was taken from the diluted mixture and applied to a poly-lysine coated cover slip, spread by pressing a slide over it. The sample was sealed with vacuum grease prior to immediate observation under a fluorescence microscope. The purpose of the dilution was so that the filaments were dispersed enough to discern those with branches, not those simply appearing to overlap with each other in a crowded field of view. Even in these diluted samples, slight overcounting was not totally prevented, especially when filaments grew to microns in length. On the other hand, the dilution step might have disrupted some filament clusters, thus the images we acquired tend to under-represent the actin branches or clusters existing in the initially more concentrated samples. These two opposite effects were both neglected as the filaments with and without branches were counted.

C. Light scattering

Static light-scattering signal was conveniently detected at a fixed scattering angle of 90° using a Perkin Elmer LS-5B luminescence spectrometer, as described previously [16]. The wavelength of the incident light was set at 365 nm. It ought to be noted that the scattering intensity recorded using such an instrument lacks the sensitivity and technical refinements for determining the average molecular or cluster size of the test sample. Instead, the instrument only serves the purpose of reliably monitoring changes in scattering of a fixed sample over time or detecting differences among samples read under a fixed setting of wavelength, slit width, sample size and geometry, etc. The intensity readings acquired are presented customarily with arbitrary units, although in actual fact they were recorded in voltage after the scattered-light signal was amplified and converted into electric signal by the spectrometer. Nevertheless, the setup is suitable for the study of polymerization kinetics and the relevant time constants can be obtained from fitting the scattering intensity curves independent of the actual values of the light intensity.

III. RESULTS

We first sought visual evidence of the effects of Arp2/3 on actin polymerization by the commonly employed technique of fluorescence microscopy. The accepted notion is that the purified Arp2/3, upon activation by the soluble, constitutively active VCA domain of N-WASP, nucleates new actin filaments from the side of existing ones, thereby stimulating actin assembly. To visualize branch formation, TRITC-phalloidin was added into the protein mixture at selected times following the initiation of the actin assembly. Numerous actin filaments were observed within minutes. Even with a dilution of 20–50 fold in order to discern dispersed filaments with limited optical resolution, an estimated 10%–20% of filaments showed branched structures 2 to 3 min into the polymerization, as seen in Fig. 2(a). At about 6 min, the percentage of branched filaments peaked at over 40%. After that, the number of branched filaments decreased over time. This observation confirms the results of previous kinetic studies [17,18]. It is qualitatively consistent with the theoretical prediction of the autocatalytic polymerization theory [13], where the term "branching explosion" was introduced to describe the rapid growth of actin filaments in highly branched clusters seen in simulations. In our microscopy observation, we did notice small clusters of branches, but this assay underestimated branches for at least three reasons: First, branches on the order of 100 nm or shorter were not detected due to limited optical resolution. Second, dilution of the sample and adsorption of branched filaments to a coverslip surface might also have disrupted some clusters. Last but not least, in our simple counting scheme, a small number of long filaments with multiple branches were each counted as one branched filament, thus when numerous clusters of branches were formed they were severely underestimated. Nevertheless, the decrease in the number of actin filaments with visible branches over the time course of 30 min or longer is a reliable observation.

To independently confirm the transient assembly kinetics, we chose to perform a light-scattering study, which is less perturbative and does not rely on counting individual

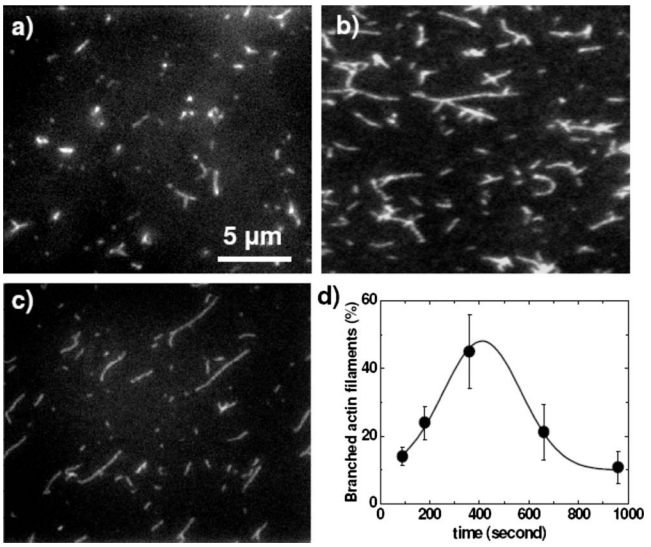

FIG. 2. Fluorescence imaging of polymerized actin at different times from the initiation of the polymerization process, shown at $t=3$ min (a), 6 min (b), and 16 min (c), respectively. A large number of branched actin filaments were noted minutes into the polymerization, but few remained over long time (d). The percentage of branched actin filaments was determined by counting over $n=170$ filaments at each selected time point. Error bars indicate standard deviation over two or three independent experiments. The concentrations of the constituent proteins were: [actin]=2 μM, [Arp2/3]=20 nM, [gelsolin]=5 nM, and [VCA]=100 nM.

branches. The autocatalytic polymerization theory [13] predicts a kinetic overshoot in light-scattering intensity during the polymerization of actin with appropriate amounts of Arp2/3 and capping proteins. This key prediction is indeed confirmed by our experiment. Figure 3 shows a scattering intensity measurement over time with a prominent overshoot. The light intensity (in arbitrary units) rises rapidly over about 20 s. It reaches a peak at ~200 sec and dips to a steady state level at later times. The light-scattering intensity reflects both the extent of polymerization and the size of the branched clusters. The rapid rise is caused by a combination of an increasing amount of polymerized actin, an increasing average filament length, and possibly an increasing number of filaments per cluster linked by Arp2/3 initiated branches. The drop, which is much slower than the rise, is probably mainly due to a drop in the average cluster size due to debranching, since no net depolymerization is expected with the presence of sufficient ATP in the actin solution, as was true in our experiment. This result agrees well with the simulation prediction (inset of Fig. 3, adopted from [13]), although the experimental data peak at a shorter time, followed by a much smaller drop in scattering intensity.

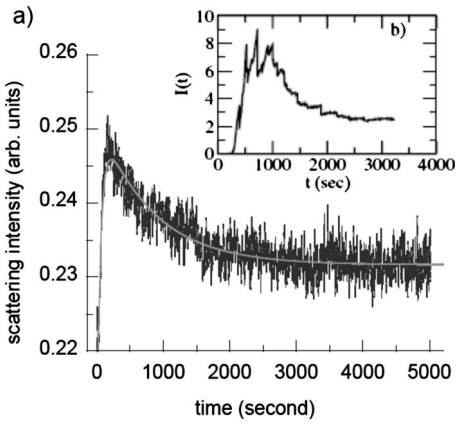

FIG. 3. Light-scattering intensity of polymerizing 2 μM actin with 15-nM Arp2/3 complex, 100-nM VCA in order to activate Arp2/3, and 5-nM gelsolin as the capping protein. The data fits well with a double exponential function, indicative of a rapid process of polymerization, followed by a slower convergence to a steady state. The slower process may be caused by debranching. Inset, simulation prediction of scattering intensity over time, for [actin] =2 μM, capping protein concentration [CP]=5 nM, and [Arp2/3]=14.3 nM (adapted from Ref. [13])

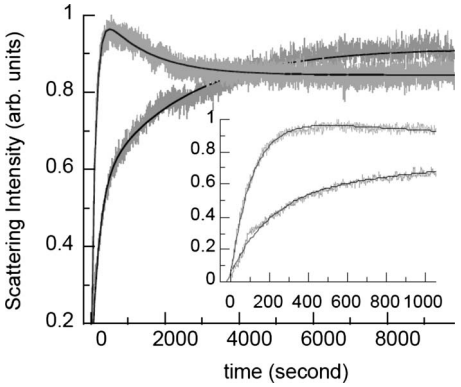

FIG. 4. Light-scattering intensities of polymerizing actin with or without branching kinetics. The lower trace is for a control sample containing 10 μM actin, showing no overshoot during the growth. The upper trace is for a sample containing 10 μM actin, 5-nM gelsolin, 14.5-nM Arp2/3 complex, and 600-nM VCA, showing an overshoot peaked at ~500 sec. Both traces are fits with double exponential functions, showing, however, dissimilar two-stage processes. Inset shows the same curves but only over the first 1000 sec.

Since the magnitude of the decreasing tail is expected to be proportional to the height of the initial peak, we use the following formula for a double exponential fit to the scattering intensity curves:

$$I = p_1[1 - p_2 \exp(-t/\tau_1)][1 + p_3 \exp(-t/\tau_2)], \qquad (1)$$

where p_1, p_2, p_3, τ_1, and τ_2 are positive fitting parameters. Here, τ_1 describes the growth rate, while τ_2 is the time constant for the slow drop after the scattering intensity peaks, and is roughly the larger of τ_{dis} and τ_{depol}. The fit parameters we obtain are $\tau_1=60.2$ sec and $\tau_2=809$ sec. The value of τ_2 is consistent with previous estimates of debranching rates [17,19] suggesting that debranching is the main process involved in determining this time.

Figure 4 shows a similar overshoot in a sample with [actin]=10 μM, [Arp2/3]=14.5 nM, [gelsolin]=5 nM, and [VCA]=600 nM, compared with a control polymerization curve with actin as the only protein in the sample, for which case no overshoot occurs. The large excess of VCA was added here to ensure full activation of Arp2/3. The binding rate of VCA to Arp2/3 is known to be fast, and with

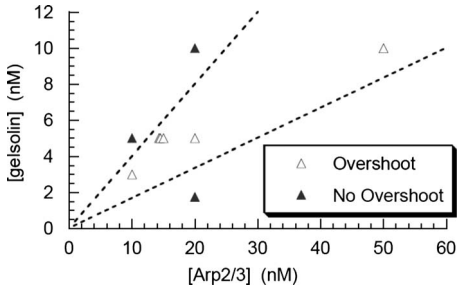

FIG. 5. Dependence of the kinetic overshoot on the concentrations of gelsolin and Arp2/3 for [actin] either 2 μM or 10 μM. The overshoots were only observed within the section where $2.5 < [\mathrm{Arp2/3}]/[\mathrm{gelsolin}] < 6$. This finding is consistent with the kinetic analysis of Ref. [13].

submicromolar affinity [20]. The double exponential fit as shown in Eq. (1) yields $\tau_1 = 116$ sec and $\tau_2 = 1255$ sec, respectively. The absence of a peak in the control sample suggests that the peak is due to branching, which is absent in the control sample.

We have tested the dependence of the kinetic overshoot on the participating proteins by performing the scattering measurements for polymerization using varying concentrations of actin, gelsolin, and Arp2/3. The overshoot phenomenon occurs at actin concentrations of both 2 μM and 10 μM, each with several different combinations of [Arp2/3] and [gelsolin], but only under the condition of $2.5 < [\mathrm{Arp2/3}]/[\mathrm{gelsolin}] < 6$ (Fig. 5). Whereas whether or not there is an overshoot at each condition tested was reliably tested, we note that the fan shaped region delineated in Fig. 5 is guided by the theoretical prediction. The conclusion of this experimental study ought to be put within the context of the limited conditions tested, and the choice of this condition set is justified further in the Discussion section.

IV. DISCUSSION

The existence of a window of values [Arp2/3]/[gelsolin] for an overshoot to occur is consistent with the kinetic analysis of Ref. [13]. Evaluation of Eq. 10 (on the cluster size) of that analysis shows that the maximum cluster size (measured in number of filaments) seen during the early stages of polymerization is $k_{\mathrm{br}} k_{\mathrm{on}} \tau_{\mathrm{dis}} / k_{\mathrm{cap}}$, where k_{br} is the branching rate per subunit of a filament, k_{on} is the on-rate, and k_{cap} is the capping rate. Thus, the branching explosion should be associated with high values of $k_{\mathrm{br}}/k_{\mathrm{cap}}$, which should in turn be associated with high values of $[\mathrm{Arp2/3}]/[CP]$. This analysis confirms the intuition that the branching explosion is caused by a relatively high concentration of the branching protein, Arp2/3, and inhibited by a high concentration of the capping protein, which tends to abolish the branch growth. At the other extreme, however, if $[CP]$ becomes so small that the filament length substantially exceeds the wavelength of the light used in the scattering experiment, an assumption used in Ref. [13], that the light scattering from a cluster is entirely in phase, becomes invalid. In this case, most of the coherence in the scattering comes from interference between subunits on the same filament, and the cluster size no longer affects the light scattering strongly. Therefore the overshoot also disappears at small values of $[CP]$, leaving a finite window where it is observed.

Recent work by Akin and Mullins examines the relationship between the branching protein complex, Arp2/3, and capping protein, in the context of cell motility [7]. Their major finding is that capping protein increases the rate of actin-based motility by promoting more frequent filament nucleation by Arp2/3 complex. Akin and Mullins measured the growth rate of actin comet tails or clouds induced by micron-sized beads coated with ActA, which stimulates actin assembly at the bead surface. Remarkably, their findings reveal a relationship between Arp2/3 and capping protein similar to ours, in the sense that either tail growth or symmetry breaking appears to occur at a particular ratio of $[\mathrm{Arp2/3}]/[CP]$. For instance, the symmetry-breaking threshold occurs at $[\mathrm{Arp2/3}]/[CP] \approx 2$, and the transition becomes sharper at higher concentrations of capping protein. The symmetry-breaking phenomenon treated in that paper is very different from the overshoot phenomenon studied here, but these results confirm the importance of the key parameter $[\mathrm{Arp2/3}]/[CP]$ introduced in Ref. [13].

An alternative explanation of overshoots like the ones seen here has been proposed recently based on the effects of ATP hydrolysis on the polymerization properties of actin [21]. In that mechanism, the concentration of polymerized actin itself displays an overshoot as a function of time. It occurs when the polymerization is fast in comparison with the nucleotide exchange time. We do not believe that this is the dominant process in the phenomena observed here. Previous experiments of actin polymerization using the pyrene assay, with protein concentrations very similar to ours [22], found no overshoot in the amount of polymerized actin. Furthermore, the time scales of the overshoots caused by ATP hydrolysis are typically less than 100 sec [23], much shorter than those seen here (809 sec and 1255 sec for the overshoot curves shown in Figs. 3 and 4, respectively). Therefore, the branching explosion followed by debranching appears to be the more plausible explanation of our results.

Another alternative explanation for the slow drop in scattering over a long time is partial depolymerization of the actin network as the amount of adenosine tri-phosphate (ATP) in the sample is gradually depleted. In our experiments, the initial concentration of ATP is set to be 0.2 mM, at 20–100 fold excess to the molar concentration of actin, which is in turn on the order of 100 fold excess to Arp2/3. The consumption of ATP by Arp2/3 can be safely neglected due to the over 10,000 fold molar ratio of [ATP]/[Arp2/3]. Since the actin polymerization causes hydrolysis of one ATP molecule per actin monomer, depletion of ATP would require 20–100 cycles of actin polymerization, the time scale of which would be longer than several hours. Furthermore, the rate of ATP depletion due to actin polymerization would be proportional to the actin concentration. Thus one would expect the time constant for a 10 μM sample to be shorter than the 2 μM actin sample. In contrast, we found the time constant of $\tau_2 = 1255$ sec for the former to be even slightly longer than 809 sec for the later. We therefore rule out this explanation.

In summary, the experimental results obtained in a reconstituted actin assembly system confirm the key predictions of

a theory proposing a branching explosion based mechanism. We showed that the number of branches per filament reaches a maximum and then decreases. We observed a characteristic overshoot in actin network assembly. Finally, we found that the overshoot occurs only in a limited range of the ratio [Arp2/3]/[gelsolin], another key prediction of the theory. Since the proteins used are among the most essential ones at the leading edge of motile cells, the results of our experimental work also provide key insights toward understanding actin dynamics in cellular settings. For instance, most cellular processes develop on the time scales of seconds or minutes, and the most relevant states of actin assembly are nearly always away from thermodynamic equilibrium. Therefore, the observed phenomena of branch explosion and kinetic overshoot are likely relevant to cytoskeletal activation and cell migration.

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[1] T. D. Pollard and G. G. Borisy, Cell **112**, 453 (2003).
[2] P. Sheterline, J. Clayton, and J. Sparrow, *Actin (Protein Profile)* (Oxford University Press, Oxford, England, 1998) 4th edition.
[3] T. M. Svitkina, A. B. Verkhovsky, K. M. McQuade, and G. G. Borisy, J. Cell Biol. **139**, 397 (1997).
[4] T. M. Svitkina, A. B. Verkhovsky, and G. G. Borisy, J. Struct. Biol. **115**, 290 (1995).
[5] T. P. Loisel, R. Boujemaa, D. Pantaloni, and M. F. Carlier, Nature (London) **401**, 613 (1999).
[6] S. Wiesner, E. Helfer, D. Didry, G. Ducouret, F. Lafuma, M.-F. Carlier, and D. Pantaloni, J. Cell Biol. **160**, 387 (2003).
[7] O. Akin and R. D. Mullins, Cell **133**, 841 (2008).
[8] R. D. Mullins, J. A. Heuser, and T. D. Pollard, Proc. Natl. Acad. Sci. U.S.A. **95**, 6181 (1998).
[9] T. P. Stossel, Science **260**, 1086 (1993).
[10] L. A. Cameron, M. J. Footer, A. van Oudenaarden, and J. A. Theriot, Proc. Natl. Acad. Sci. U.S.A. **96**, 4908 (1999).
[11] H. N. Higgs and T. D. Pollard, J. Biol. Chem. **274**, 32531 (1999).
[12] L. Blanchoin, K. J. Amann, H. N. Higgs, J.-P. Marchand, D. A. Kaiser, and T. D. Pollard, Nature (London) **404**, 1007 (2000).
[13] A. E. Carlsson, Phys. Rev. Lett. **92**, 238102 (2004).
[14] J. He, J. Viamontes, and J. X. Tang, Phys. Rev. Lett. **99**, 068103 (2007).
[15] J. X. Tang, P. A. Janmey, T. P. Stossel, and T. Ito, Biophys. J. **76**, 2208 (1999).
[16] J. X. Tang and P. A. Janmey, J. Biol. Chem. **271**, 8556 (1996).
[17] A. M. Weaver, A. V. Karginov, A. W. Kinley, S. A. Weed, Y. Li, J. T. Parsons, and J. A. Cooper, Curr. Biol. **11**, 370 (2001).
[18] L. Blanchoin, T. D. Pollard, and R. D. Mullins, Curr. Biol. **10**, 1273 (2000).
[19] K. J. Amann and T. D. Pollard, Proc. Natl. Acad. Sci. U.S.A. **98**, 15009 (2001).
[20] J. B. Marchand, D. A. Kaiser, T. D. Pollard, and H. N. Higgs, Nat. Cell Biol. **3**, 76 (2001).
[21] F. J. Brooks and A. E. Carlsson, Biophys. J. **95**, 1050 (2008).
[22] A. E. Carlsson, M. A. Wear, and J. A. Cooper, Biophys. J. **86**, 1074 (2004).
[23] L. A. Selden, H. L. Kinosian, J. E. Estes, and L. C. Gershman, Biochemistry **38**, 2769 (1999).