Under Physiological Conditions Actin Disassembles Slowly from the Nonpreferred End of an Actin Filament

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ABSTRACT Incubation of the isolated acrosomal bundles of Limulus sperm with skeletal muscle actin results in assembly of actin onto both ends of the bundles. Because of the taper of these cross-linked bundles of actin filaments, one can distinguish directly the preferred end for assembly from the nonpreferred end. Loss of growth with time from the nonpreferred end was directly assessed by electron microscopy and found to be dependent upon salt concentration. Under physiological conditions (100 mM KCl, 1 mM MgCl₂) and excess ATP (0.5 mM), depolymerization of the newly assembled actin filaments at the nonpreferred end over an 8-h period was 0.024 μm/h. Thus, even after 8 h, 63% of the bundles retained significant growth on their nonpreferred ends, the average length being 0.21 μm ± 0.04. However, in the presence of 1.2 mM CaCl₂, disassembly of actin monomers from the nonpreferred end increased substantially. By 8 h, only 7% of the bundles retained any actin growth on the nonpreferred ends, and the depolymerization rate off the nonpreferred end was 0.087 μm/h. From these results we conclude that, in the absence of other cellular factors, disassembly of actin subunits from actin filaments (subunit exchange) is too slow to influence most of the motile events that occur in cells. We discuss how this relates to treadmilling.

In the presence of ATP and salt, actin self-associates to form filaments in a reaction that involves nucleation and elongation stages (11). While elongation occurs at both ends of a filament, growth is biased, with monomers adding to one end at a faster rate than to the other (2, 13). The two ends have been identified by decoration with subfragment 1 of myosin; the “barbed” end is the preferred end for assembly and the “pointed” end is the nonpreferred end for actin monomer addition (21). Wegner in 1976 (19) theorized that, since actin filaments are polar, each end must represent a different equilibrium, so that at steady state there could be a flux of monomers through the polymer. This flux, known as “treadmilling” or “head-to-tail polymerization,” would occur in the presence of excess ATP and requires that the critical concentrations at the two ends of the filament be different from one another. More recently, Wegner and Neuhaus (20) have shown that subunit exchange is strongly dependent on the concentration and type of cations present. For example, within 3–4 h, nearly complete exchange of actin monomers into filaments takes place when actin is preassembled in 1.2 mM CaCl₂. They interpreted these results as representative of treadmilling. In the presence of KCl, however, no exchange of monomer is observed within 15 h. Margolis and Wilson (9), using pulse-chase experiments with podophyllotoxin, which blocks microtubule assembly at steady state, have demonstrated that in vitro treadmilling of microtubules may also exist.

That treadmilling may play a role in the control of sites of microtubule and microfilament assembly in vivo has been proposed by Kirschner (6). He predicts that when the critical concentration drops lower than the critical concentration at steady state, monomers will disassemble from the high critical concentration ends of filaments free in solution more rapidly than monomers will add to the low critical concentration ends. Filaments free in solution will gradually disappear and new filaments will not form. Filaments with their high critical concentration ends blocked, however, will be stable or grow until the monomer concentration is reduced to a new steady state, in equilibrium with the low critical concentration ends. Although these ideas are exciting, nevertheless, there is serious question as to just how much treadmilling exists or could exist in vivo.

Recently, Pardee et al. (12) examined actin subunit exchange under physiological conditions using both fluorescence energy transfer techniques and incorporation of 35S-actin monomer into unlabeled filaments. They showed that subunit exchange under physiological salt conditions (100 mM KCl, 1–5 mM MgCl₂ and ATP) occurs only to a small and limited extent. This same limited exchange was found for actin in 2.5 mM MgCl₂ and 0.5 mM ATP. In contrast to these results, Wang and Taylor (18), using similar procedures, found high subunit exchange in 2.5 mM MgCl₂ and 0.5 mM ATP.

A new method is now available with which to observe actin
assembly directly. It involves the use of cross-linked actin bundles, isolated from Limulus sperm, which serve as nuclei for actin growth. Within the Limulus sperm resides a bundle of highly cross-linked and unidirectionally polarized actin filaments (15). This bundle, 50 μm in length, contains 85-100 filaments at one end and gradually tapers to 13 filaments at the other end (17). These bundles can be easily isolated from the sperm and sheared into small segments ranging from 5 to 20 μm in length; they are completely stable in solution. Incubation of these segments with G-actin results in actin assembly at both ends of the bundles under appropriate salt conditions. The amount of new growth is readily recognizable because the newly assembled actin, lacking the bundling proteins present in the Limulus sperm, splays out from the ends of the segment or nucleus. The assay is very sensitive; growth of as few as 10 monomers in length can be detected. The polarity of new growth is easily learned by measuring the diameter of the bundle at each end since the bundles exhibit taper; the thinner end corresponds to the preferred or barbed end (17).

Recently, this assay has been used to examine the effects of Thyone sperm profilin on actin assembly (16). We found that profilin serves to restrict actin growth to the preferred end of the bundles. During review of our paper, James Spudich (Stanford University School of Medicine) suggested that this assay might provide a simple and direct way to visualize actin monomer flux. He pointed out that if treadmill of microfilaments exists, we would expect to see new growth being lost with time from the nonpreferred end (the thicker end of the Limulus bundle) since Wegner’s hypothesis predicts a loss of subunits (monomers) from the nonpreferred end and a concomitant addition to the preferred end of the filaments. Unfortunately, the limitations of this assay allow us to examine, at steady state, only the net disassembly of subunits at the nonpreferred end without showing a concomitant growth at the preferred end. Nevertheless, what we find is that the net disassembly of subunits from the nonpreferred end under physiological conditions is a very slow process (yet somewhat faster when actin assembly is initiated by 1.2 mM CaCl₂). Consequently, if treadmill occurs in actin filaments, under physiological conditions, it must have a very limited rate—too slow to account for control of motile processes in vivo.

MATERIALS AND METHODS

Organisms: Limulus polyphemus were collected by the Marine Resources Department at the Marine Biological Laboratory in Woods Hole, MA and kept in Instant Ocean Aquaria (Instant Ocean Aquarium Systems, Eastlake, OH) at the University of Pennsylvania.

Isolation of the Acrosomal Process of Limulus Sperm: Limulus sperm were collected and the acrosomal bundles were isolated according to Tilney (15).

Preparation of actin: Rabbit skeletal muscle actin was prepared in the laboratory of Dr. Anne Marie Weber (University of Pennsylvania) according to Spudich and Watt (14) and further purified by G-150 Sephadex chromatography according to MacLean-Fletcher and Pollard (8). Column buffer consisted of 5 mM triethanolamine, pH 8, 0.5 mM ATP, 0.2 mM CaCl₂, 1 mM NaNO₃, and 0.1 mM CaEDTA. Actin concentration was determined photometrically at 290 nm using an extinction coefficient of 24,900 M⁻¹ cm⁻¹ (19).

Actin Assembly Assay: Gel-filtered G-actin was diluted (over eightfold) to 0.2 mg/ml in buffer containing 5 mM Tris, pH 8.0, 0.5 mM ATP, and 1 mM MgCl₂. The actin rapidly assembled onto both ends of all the actin filaments, i.e., monomers being lost from the nonpreferred end and added to the preferred end (or to other filaments). Unfortunately, it is nearly impossible under the conditions we used to measure accurately the extra growth on the preferred end. This is due to the difference in rate of assembly at the two ends. To achieve measurable growth at the nonpreferred end so that disassembly can be monitored, the concentration of actin used results in extensive growth at the preferred end as well. Consequently, the newly assembled fila-
ments at the preferred end grow very long and splay apart making an accurate measure of their average growth impossible. However, the conditions are set such that the net rate of disassembly at the nonpreferred end can be measured by loss of growth with time (Table I).

![Image](image1.png)

**Figure 1** *Limulus* acrosomal bundles were added to a solution of G-actin at 0.2 mg/ml in 5 mM Tris, pH 8.0, 0.5 mM ATP, and 1% NaN₃, and the salt concentration was increased to 100 mM KCl and 1 mM MgCl₂. At specific times, a sample was negatively stained. Assembly occurs at both ends of the bundle (arrows) although more growth is seen on the apical end which corresponds to the “barbed” or “preferred” end for assembly. Bar, 1 μm. X 16,625.

| Time | Preferred, only | Neither | Total |
|------|----------------|---------|-------|
| 2 min| 100*           | 0       | 100   |
| 1 h  | 97             | 3       | 100   |
| 4 h  | 83             | 7       | 100   |
| 6 h  | 77             | 23      | 100   |
| 8 h  | 63             | 30      | 100   |

*Number of bundles = 30.

In the presence of 100 mM KCl and 1 mM MgCl₂, by 2 min actin assembled onto both ends of 100% of the bundles sampled. The mean amount of actin assembled at the nonpreferred end is 0.4 μm ± 0.05 in length (Fig. 2a and Table II). One h after initiation of actin assembly, 97% of the bundles still have growth on both ends. Slightly less growth is now found at the nonpreferred end; the average length is 0.34 μm ± 0.04 (Fig. 2b). By 4 and 6 h, (Fig. 2, c and d) the percentage of bundles with growth on the nonpreferred end drops slightly to 83% and 77%, respectively. After 1 h, no change in average length of the filaments at the nonpreferred end is detected until 6 h where μm = 0.23 ± 0.03. Even after 8 h (Fig. 2e), 63% of the bundles still have growth on both ends: average length = 0.21 μm ± 0.04. The average rate of monomer loss from the nonpreferred end is 0.024 μm/h for the 8-h time period. From the micrographs presented in Fig. 2, one gets the impression that not all the newly assembled filaments shorten at exactly the same rate since the later time points sometimes show less than the number of filaments that comprise the bundle. Likewise at early time points we have observed instances in which, at the preferred end of the bundle, a few of the filaments are much longer than the others. We interpret both these cases as merely indicating that local variations exist and that we are looking at a range of lengths that follows a statistical distribution.

When actin assembly was initiated by the addition of CaCl₂ to 1.2 mM rather than by KCl and MgCl₂, after 2 min growth occurred on both ends of only 70% of the *Limulus* bundles (Table III). Again, assembly was biased with the thicker end of the bundles having less new growth than the thinner ends (not shown). At 2 min the CaCl₂ samples averaged 0.13 μm ± 0.01 in length at the nonpreferred end (Fig. 3a and Table IV). Unlike the previous case, growth of actin at the nonpreferred end was slower and continued after 2 min. That assem-
Limulus acrosomal bundles were incubated at room temperature with G-actin at 0.2 mg/ml in 5 mM Tris, pH 8.0, 0.5 mM ATP, and 1% NaN₃, and the salt concentration was increased to 100 mM KCl and 1 mM MgCl₂. At indicated times, samples were negatively stained and assessed for whether or not assembly occurred at the nonpreferred (thicker) end of the bundles. a, 2 min; b, 1 h; c, 4 h; d, 6 h; e, 8 h. × 99,150.

**DISCUSSION**

The *Limulus* bundle assay has been used to measure directly the assembly and disassembly of microfilament ends. The method was first used by Bonder and Mooseker (2) to examine the role of villin on actin assembly and resembles that used by Bergen and Borisy (1) to assay growth of tubulin from flagellar axonemes and that used by Pollard and Mooseker (13) to assay actin assembly off microvillous cores. The advantages of using this assay are: (a) the ease with which new actin growth can be distinguished from the nuclei, i.e., the acrosomal bundles, (b) the sensitivity of the assay since growth by as little as 10 monomers can be detected, (c) the fact that the bundles are tapered, which readily allows one to discern the preferred (thinner) end for actin growth from the nonpreferred (thicker) end. More importantly, however, is the slow assembly of actin in the presence of CaCl₂ has been shown previously by Mihashi and Ooi (10). At 1 h, 63% of the bundles had growth on both ends; mean = 0.26 μm ± 0.03 (Fig. 3b). (Nearly identical growth is found at 15 min [data not given]). By 4 h, only 17% of the bundles showed any growth on the nonpreferred end; the majority lacked growth on that end (Fig. 3c). By 6 h, not only was growth absent from the nonpreferred end (Fig. 3d), but 57% of the bundles lacked growth on the preferred end as well. The reason for this is not clear to us. Similar results are found at 8 h (Fig. 3e). Rate of growth, then, in the presence of 1.2 mM CaCl₂, is slower than in 100 mM KCl and 1 mM MgCl₂ and is followed by disassembly at 0.09 μm/h from the first to the fourth hour.

**TABLE III**

Percentages of Bundles Exhibiting New Actin Growth onto *Limulus* Bundle Ends after Initiation of Assembly with 1.2 mM CaCl₂

| Time | Both | Preferred only | Neither | Total |
|------|------|----------------|---------|-------|
| 2 min | 70* | 30 | 0 | 100 |
| 1 h   | 63   | 37 | 0 | 100 |
| 4 h   | 17   | 43 | 40 | 100 |
| 6 h   | 10   | 33 | 57 | 100 |
| 8 h   | 7    | 27 | 66 | 100 |

* Number of bundles = 30.

**TABLE IV**

Average Lengths of New Actin Growth onto Nonpreferred Ends of *Limulus* Bundles after Initiation of Assembly with 1.2 mM CaCl₂

| Time | Length | No. of bundles | % of bundles with no growth |
|------|--------|----------------|----------------------------|
| 2 min| 0.13 ± 0.01 | 18 | — |
| 1 h  | 0.26 ± 0.03 | 13 | — |
| 4 h  | —      | —  | 83 |
| 6 h  | —      | —  | 90 |
| 8 h  | —      | —  | 93 |
FIGURE 3  *Limulus* bundles were incubated with G-actin at 0.2 mg/ml in 5 mM Tris, pH 8.0, 0.5 mM ATP, and 1% NaN₃, and the actin assembly was initiated by adding CaCl₂ to 1.2 mM. At indicated times, samples were negatively stained and assessed for whether or not assembly occurred at the nonpreferred end of the bundles. a, 2 min; b, 1 h; c, 4 h; d, 6 h; e, 8 h. × 99,150.

ferred (thicker) end for assembly, without the use of heavy meromyosin or subfragment 1 decoration of the filaments, (d) the stability of the bundles that, unlike the microvillous cores, do not fall apart in solution, and (e) the directness in interpretation of results.

It is clear that the net rate of disassembly from the nonpreferred end is a function of the ionic concentration. Loss of growth from the nonpreferred end is very slow under physiological salt conditions (100 mM KCl, 1 mM MgCl₂) and when ATP is not limiting, however, it is somewhat faster in 1.2 mM CaCl₂. This result is consistent with those of Wegner and Neuhaus (20) who demonstrated that in 19 mM KCl, no exchange was observed within 15 h, whereas fast exchange could be initiated with addition of CaCl₂ to 1.2 mM. Likewise Wang and Taylor (18), using a fluorescence energy transfer assay, found that actin at 0.2 mg/ml showed a sixfold decrease in the rate of subunit exchange at 1 h when assembly was promoted by 100 mM KCl and 1 mM MgCl₂ in 1 mM ATP rather than 2.5 mM MgCl₂. And more recently, under conditions similar to ours (0.2 mg/ml actin, 100 mM KCl, 1 mM MgCl₂), Pardee et al. (12) also found no more than 10–20% exchange.

The behavior of actin under the CaCl₂ conditions is clearly different from that under physiological salt conditions. Not only is loss of growth from the nonpreferred end faster in CaCl₂, but the rate of assembly is much slower. At 2 min the average length of newly assembled actin approaches only 25% of that found at 2 min under the KCl, MgCl₂ conditions. Additionally, the extent of assembly never reaches that found in the KCl, MgCl₂ samples. That assembly of actin proceeds more slowly in CaCl₂ than in MgCl₂ has been previously observed; however, the final degree of polymerization as measured by viscometry reached the same value in each case (10). More perplexing, however, is why with time there is a loss of growth from both the nonpreferred ends and the preferred ends of the bundles. This result was also found using actin at 0.1 mg/ml. James Spudich has offered the explanation that this result may be due to magnesium being bound to its site on the actin molecule. When the 1.2 mM calcium is added, assembly is initiated because of the high amount of divalent cation present; the critical concentration would be <0.2 mg/ml because of magnesium's presence. However, the calcium would slowly (over hours) displace the magnesium and the critical concentration would rise above 0.2 mg/ml, resulting in disassembly from both ends of the filaments.

The experiments reported here show that under physiological conditions the net disassembly of monomers from the nonpreferred end is very slow. Unfortunately, because of the excessive growth and splaying apart of the filaments at the preferred end of the bundle at this actin concentration, we are unable to determine if these filaments increase in length by an amount comparable to the loss at the nonpreferred end, or, in other words, if treadmilling is occurring. Thus, it is not possible from our experiments to distinguish treadmilling from other mechanisms that would involve loss of subunits from the nonpreferred end. There are two such mechanisms: (a) a redistribution of monomer between filaments and the nonfilamentous steady state pool of actin, and (b) a redistribution of monomers from bundle-nucleated filaments to filaments assembled without bundles. For mechanism (a), a net
depolymerization of actin would have to occur. If such a net depolymerization were occurring for some reason such as denaturation of the actin in the subunit pool, then the measured disassembly of monomers from the nonpreferred end would, in reality under “more ideal conditions”, be even slower than that measured in this report. As will be discussed in more detail subsequently, it is already too slow to play any important physiological role in motile processes; if slower, then this conclusion is just reinforced. For mechanism (b) to occur, a process often referred to as actin filament length redistribution, Oosawa and Asakura (11) have shown that two days are required. Clearly our rates of disassembly are faster than this, but, here again, it does not affect our conclusion that the disassembly of monomers from the nonpreferred end is very slow, a conclusion that is particularly significant in regard to recent hypotheses on the role that monomer flux might play in vivo. Let us be more specific: We often find actin filaments in cells that are ~1 μm in length. An obvious example is microvilli. Suppose that a cell wants to eliminate its microvilli and the actin filaments within them by allowing the actin to disassemble from its nonpreferred end. To make the calculations simpler, we will cap the preferred end. From our data we calculate that a 1-μm long microfilament capped at its preferred end would require >40 h to disassemble from its nonpreferred end. If the preferred end of the filament is not capped, the required time would exceed 40 h because, although disassembling from its nonpreferred end, it would simultaneously be growing at the preferred end! Thus, if treadmilling or subunit loss from the nonpreferred end, under physiological conditions, irrespective of the reason, is as slow as our work suggests, its role in many biological processes that would require fast exchange, e.g., cytokinesis, acrosomal re-actions, phagocytosis, pseudopod formation, and ruffling of cell membranes, would be insignificant because the time course of all these reactions is a few minutes, not many hours.

In a similar fashion it has been theorized by Hill and Kirschner (5) that free filaments are unstable relative to anchored filaments and so with time will disassemble by treadmilling. This would be biologically useful because in order for the cell to maintain its proper morphology or to function in motility and intracellular transport, the microfilaments must be precisely arranged. Spontaneous assembly of filaments would serve to disrupt the order, hence the ability of the cell to monitor assembly is imperative. Over the 8-h period examined, we have not found that the number of background filaments (data not shown) changes significantly. As a result, treadmilling alone, or loss of monomers from the nonpreferred end of the filament, appears to be too slow to eliminate the unanchored filaments.

While it seems that monomer flux of actin alone is insignificant, it is clear that the cell possesses the ability to modify the flux rate, i.e., either to speed it up when a fast turnover rate would be advantageous or to slow it down and thereby limit flux. Increasing evidence points to the fact that actin is intricately regulated by a host of various accessory proteins, making it highly likely that actin-binding proteins in all biological processes act to regulate actin assembly, for example, by monitoring concentration, directing growth, and by acting as filament length regulators. Conceivably, proteins are also present that serve to promote filament disassembly and enhance subunit exchange. Such a role has been assigned to profilin, a protein isolated from the slime mold, Physarum. This protein drastically reduces the viscosity of F-actin solutions (4). Another actin-regulatory protein, this one isolated from Dictostelium, is called severin. In the presence of calcium, severin binds intact filaments, and within 30 sec the filaments are completely fragmented into small pieces. The fragments then partially depolymerize, causing an increase in flux (22). Other accessory proteins act as cappers or end-blocker proteins. These proteins affect actin assembly by binding tightly to one end of the filaments. They include macrophegase gelsolin, Acanthamoeba capping protein, and β-actin (for a review of actin-binding proteins, see references 3 and 7). Clearly, one way to stop monomer flux entirely or at least to limit it is to cap the end(s) so that a static filament results. For example, the actin filaments in skeletal muscle turn over about every 30 d. In these cells there should be a capper on the pointed ends to inhibit monomer flux out of the filament and at the same time a second capper on the preferred ends to inhibit filament elongation.

We would especially like to thank Dr. James Spudich for suggesting to us that the Limulus assay could be used to examine treadmilling. Moreover, we are particularly grateful to Dr. Spudich and the reviewers of this paper for their more than helpful comments which greatly improved it. We thank Dr. Annamarie Weber and Dr. Neville Kal- lenbach for discussions and Doug Wray for assistance with the working prints.

This work was supported by National Institutes of Health HD 14474 to L. G. Tilney.

Received for publication October 4, 1983, and in revised form 4 August 1983.

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