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Assembly and Dynamics of the Actin Filament System in Nonmuscle Cells

Thomas D. Pollard
Department of Cell Biology and Anatomy, Johns Hopkins Medical School, Baltimore, Maryland 21205

Kinetic analysis has provided a detailed quantitative description of the mechanism of actin polymerization as well as the methods to analyze the mechanisms of action of actin-binding proteins. In Acanthamoeba, five different proteins regulate the pool of monomers available for polymerization, cap the end of filaments, sever filaments, and cross-link filaments. Remarkably, many of these interactions involve very-low-affinity bonds between the protein molecules.

Key words: actin, actin-binding proteins

Recent studies on the assembly of actin filaments and the regulation of this process by a variety of actin-binding proteins have provided a number of potentially important new insights into the assembly and dynamics of the actin filament system in nonmuscle cells. This paper uses data from our work on actin and actin-binding proteins of Acanthamoeba to illustrate the following points: (1) Low-affinity interactions between structural proteins are important and in fact essential for some functions of the actin filament system. The examples include: very weak bonds between the subunits in actin dimers and trimers on the pathway to nucleus formation, weak bonds between actin filaments and the crosslinking protein alpha-actinin; and weak bonds between capping protein and small actin oligomers that very effectively promote the formation of nuclei. (2) Apparently simple structural proteins can have very sophisticated mechanisms of action. In the Acanthamoeba system, actin monomer binding proteins, capping protein and crosslinking proteins all have surprisingly complex mechanisms of action. (3) Kinetic analysis of individual reactions is necessary to understand complex mechanisms. My best example is the process of actin polymerization where four different steps are necessary to form a filament and where at steady state no less than 10 different reactions establish the steady state monomer concentration. (4) Mechanical analysis of cytoskeletal proteins is necessary to understand their real functions. This point might, at the outset, appear to be obvious, but relatively little has yet been learned about the actual mechanical properties of cytoskeletal proteins in spite of the importance of these properties to their functions in the cell.

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(5) Most of the components of the actin regulatory system are universal. Several years ago this assertion was not at all obvious, because the inventory of actin binding proteins in any given cell type was far from complete. Enough work has now been done that we can see (Table I) that most of the different classes and families of actin binding proteins are found in enough different cell types to draw the conclusion that most of these proteins have homologs in many, if not all cell types.

ACTIN ASSEMBLY

For years it has been known that actin filaments assemble from monomers in a process that involves at least two steps [1]. First, there is a slow, rate-limiting step during which small oligomers called *nuclei* are formed. These nuclei then elongate rapidly to form long actin polymers. Detailed presteady-state kinetic analysis by three different laboratories [2–4] has established that there are actually three or four steps in this process. Further, it has been possible to estimate the absolute values for the rate constants for each of these steps and thereby to write a fairly complete mathematical description of the assembly process including the rates of the various reactions. The method used to obtain this information involved several steps. First, we measured the absolute values of the elongation rate constants by electron microscopy [5]. Second, we measured the dependence of the full time course of polymerization on a wide range of concentrations of actin monomers. A computer then fit this data to mathematical models for the polymerization process. The variables were the size of the nucleus and the rate constants for activation, nucleation, and fragmentation. Remarkably, for such a complex process, it is possible to obtain an excellent fit between the mathematical model and the experimental data with one nucleus size and a single set of rate constants.

The overall mechanism of assembly is summarized in Figure 1. Beginning with actin monomers having bound ATP and Ca^{2+}, nuclei form by two highly unfavorable association reactions leading to a trimer. The trimer is defined as the nucleus because it elongates at the rapid rate characteristic of the elongation of larger polymers. In contrast, the reactions leading to the trimer are highly unfavorable and are the rate-limiting steps in the whole process. Using a steady-state assumption [6] in which the

TABLE I. Distribution of Actin-Binding Proteins

Class	Subunit MW (kDa)	Acanthamoeba	Other lower eukaryotes	Vertebrates
Spectrin	220–260	+	+	+
ABP/filamin	250	0	0	+
Myosin/myosin-II	175–200	+	+	+
Myosin-I	125–130	+	+	0
Vinculin	~140	0	0	+
α-Actinin	90–100	+	+	+
Gelsolin	90	0	0	+
Accumentin	65	0	0	+
Fragmin/severin	40–42	0	+	0
Capping protein	29+31	+	+	+
Gelactin-I–IV	23–38	+	0	0
Actophorin/depactin	17	+	+	+
Profilin	12–15	+	+	+

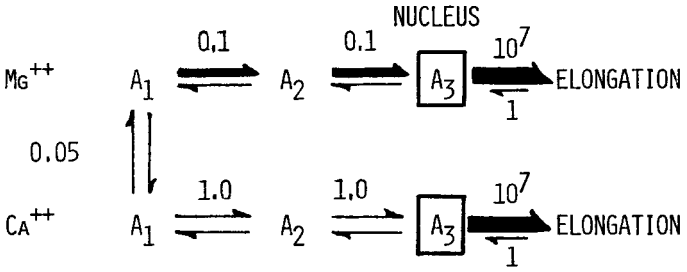

Fig. 1. Mechanism of actin polymerization. Actin monomers (A_1) form dimers (A_2) and trimers (A_3) slowly and then rapidly elongate into large polymers. The single numbers over the nucleation reactions are dissociation constants in moles liter^{-1}. The numbers over the elongation reactions are association constants with units of $M^{-1}sec^{-1}$ and dissociation constants with units of sec^{-1}. The upper line corresponds to the reactions in the presence of 50 - 100 mM KCl with millimolar Mg^{2+}. The lower lines are the reactions in 50–100 mM KCl with millimolar Ca^{2+}. The transition from Ca^{2+} to Mg^{2+} is a slow reaction with an apparent first-order rate constant of about 0.05 sec^{-1}.

monomer to dimer and the dimer to trimer reactions have the same equilibrium constant, both the dimer and trimer appear to have dissociation constants in the range of 0.1–1M. Consequently, in this assembly reaction the two most important reactions depend on very weak interactions between the protein subunits. It is important to note that the trimer nuclei are present in very low concentrations for two reasons: First, trimers are very unstable, rapidly dissociating a subunit to become dimers; second, they are rapidly consumed by elongation to form longer filaments. Consequently, the life time of trimers is probably measured in milliseconds in most experiments.

When actin monomers with bound Ca^{2+}, are polymerized in the presence of millimolar concentrations of Mg^{2+}, there is a slow, rate-limiting, first-order reaction [2] that is most likely, according to the work of Frieden [4,7], the exchange of bound Ca^{2+} for Mg^{2+}. This is the so-called activation reaction, because actin having bound Mg^{2+} forms nuclei and elongates filaments more rapidly than actin monomers with bound Ca^{2+}. In Mg^{2+}, the nucleus is again a trimer, but the reactions leading to the formation of the trimer are more favorable than for unactivated monomers. Nonetheless, the dissociation constants for the dimer and trimer species are still 100 mM according to this analysis.

Once nuclei are formed, filaments can grow very rapidly because of the large absolute value of the association rate constant for elongation at the barbed end, 10^7 $M^{-1}sec^{-1}$ [5]. This value suggests that the process is limited by diffusion. The association rate constant at the pointed end of the filament is about five or ten times smaller, but this is still a very rapid reaction. The dissociation rate constants at the two ends are on the order of 1–2 sec^{-1}. Another important reaction during elongation is the hydrolysis of ATP bound to the subunits within the filament [8]. This appears to be a first-order reaction with a rate constant of about 0.05 sec^{-1} [9,10].

Knowing all these rate constants, one can predict what happens during elongation of actin filaments. For example, if a nucleus were provided with 10 μM actin subunits, it would elongate very rapidly. In 1 sec, 100 subunits would add at the barbed end in addition to about ten subunits at the pointed end. In this single second, only one subunit would dissociate from each end, so the process is highly efficient. Since the ATP hydrolysis rate constant is small, a minority of these newly added actin

subunits would hydrolyze their bound ATP during this second, leading to a long stretch of ATP-containing subunits at both ends of the polymer.

At steady state, it is more difficult to predict what happens, because there are so many different reactions involved (Fig. 2). Were only barbed ends available for a reaction with ATP-containing subunits, rate constants measured in initial rate experiments predict that the subunit concentration would fall to 0.1 μM at steady state with one ATP-subunit associating and one ATP-subunit dissociating every second. Were only pointed ends available, the rate constants predict that the steady-state monomer concentration would be 0.5 μM and that one ATP-subunit would add and one ATP-subunit would be lost from this end each second. In that these two predictions are different, we expect that the actual steady-state monomer concentration will fall between these two values. Consequently, there will be a flux of subunits through the filaments with a small net addition of actin monomers at the barbed end balanced by an equal rate of subunit loss from the pointed end of the filament. To complicate the situation, the rate constants for association and dissociation of ADP actin are different from those for ATP actin [10,11]. Although the known rate constants suggest that most of the actin subunits at the ends of the filament will have bound ATP, we can expect that occasionally the terminal subunit will have bound ADP because of hydrolysis there or dissociation of terminal ATP subunits. When this happens, this ADP subunit will dissociate at a much higher rate than an ATP actin subunit, and, if

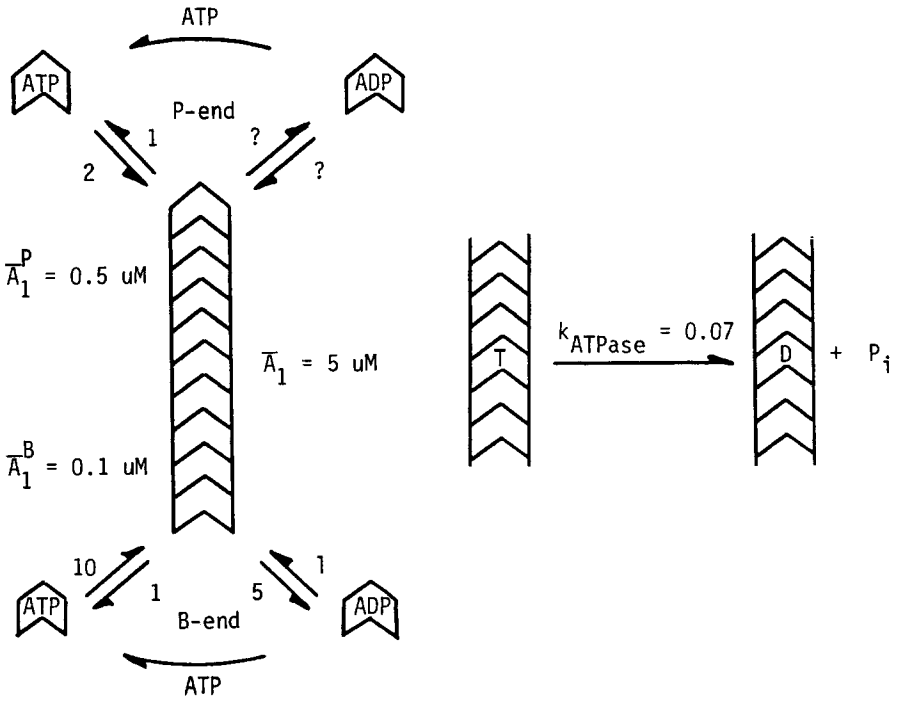

Fig. 2. Steady state reactions of actin monomers with actin filaments. The association rate constants have units of $\mu M^{-1} sec^{-1}$. The dissocation rate constants have units of sec^{-1}. $\bar{A}_1$ represents the critical concentration for the various reactions.

internal subunits also have bound ADP, then that filament will shorten rapidly for a brief period of time until an ATP-containing subunit binds to the end of the filament and stabilizes it.

This situation differs considerably for microtubules. There, the very large off rate for GDP-tubulin and the existence of multiple subunit association/dissociation sites at the polymer end lead to "phase changes" at steady state. Occasionally, a microtubule will expose GDP-subunits at the end and then it will rapidly depolymerize (usually completely) providing subunits that allow the remaining microtubules with GTP subunits at their ends to continue growing slowly. These phenomena are well documented [12], but the individual rate constants are not as fully established as for actin.

This analysis of actin assembly is interesting in its own right, because actin assembly has been one of the classic examples of self-assembly in biology, but the analysis has served another important function. It has provided methods to analyze the various steps in the assembly process. These methods are necessary to evaluate the detailed mechanisms of action of the various actin-binding proteins.

ACTIN-BINDING PROTEINS

Rather than attempt a review of the literature on actin-binding proteins, five different actin-binding proteins that we have studied from *Acanthamoeba* are used as examples (Table II). Each of these proteins has homologs in other lower eukaryotes as well as in vertebrates, so we expect that they are universal cellular constituents.

Profilin [13] is a low-molecular-weight actin monomer-binding protein found in high concentrations in the amoeba [14]. The ratio of profilin to actin is about 1:2. Profilin inhibits actin polymerization from monomers in a concentration-dependent fashion. Profilin also binds to actin monomers with a dissociation constant of ~ 5–10 μM as estimated by three independent procedures. Its effect on nucleation has been assessed by the computer curve-fitting methods that were used to study actin nuclea-

TABLE II. Actin System of Acanthamoeba

Class	Subunit composition (kDa)	Content (μmole/kg)	Molar ratio
Actin	1 × 42	170	100
Monomer binding proteins			
Profilin	1 × 12	100	59
Actophorin	1× 17	24	14
End binding protein			
Capping protein	29 + 31	2.1	1
Cross-linking proteins			
Gelation protein	2 × 90	4.2	3
Spectrin	? × 260	0.2	<1
Gelactin-I	23		
Gelactin-II	28		
Gelactin-III	32		
Gelactin-IV	38		
Myosins			
Myosin-I	1 × 125–130	1.3	<1
Myosin-II	2 × 175	2.3	1

tion, leading to the conclusion that free actin but not actin-profilin complexes can participate in the nucleation process [15,16]. Effects of profilin on elongation might be more complex. By electron microscopy, only free actin monomers appear to add to the pointed end of growing filaments [15]. On the other hand, the elongation rate at the barbed end is far higher than expected given the concentration of free actin monomers. This led to the suggestion that not only actin monomers but the complex of actin with profilin can bind to the barbed end of the filament. According to this model, profilin bound to the end of a filament acts as a capping protein preventing the addition of subsequent monomers, but the binding is so weak to the filament that it rapidly dissociates and thus has only a small effect on the elongation process [16]. These properties of profilin appear to be useful for the cell. First, most of the unpolymerized actin in the cell would be bound to profilin. This would strongly suppress spontaneous nucleation and allow the cell to specify, through the nucleating proteins that it has available, the time and place for actin assembly without being confused by spontaneous formation of filaments from actin itself. On the other hand, in that both free actin and actin-profilin complexes appear to be capable of binding to the barbed end of actin filaments, whenever a free barbed end is available, there would be a substantial pool of subunits available to promote elongation at that end.

Acanthamoeba actophorin [17] is a small molecule consisting of a single 15,000 MW polypeptide that might be related to brain actin depolymerizing factor [18] in echinoderm depactin [19]. Actophorin inhibits the extent of actin polymerization and the rate of elongation at both ends of filaments in a concentration-dependent fashion. Paradoxically, it also accelerates the spontaneous polymerization of actin from subunits. These two effects are explained by the ability of actophorin to form a nonpolymerizable complex with actin monomers under polymerizing conditions and its ability to sever actin filaments into short pieces. The severing of spontaneously formed actin filaments leads to an increased concentration of ends available for growth, so, even though part of the actin molecules are tied up in nonpolymerizable complex, the bulk rate of polymerization is increased. The function of actophorin in the cell is not known, but together with profilin the actophorin accounts for most of the unpolymerized actin found in the cell [20,21]. It also has the potential for regulating the length of actin filaments.

Capping protein [22] is a heterodimer consisting of immunologically distinct 29,000 and 31,000 MW polypeptides [23]. It received its name because it blocks the addition of subunits at the barbed end of actin filaments. Consequently, under conditions in which the critical concentrations differ at the two ends, capping protein shifts the critical concentration from a value close to that of the critical concentration at the barbed end to the value of the critical concentration at the pointed end [23]. Although capping protein eliminates $> 90\%$ of the elongation reaction by blocking the barbed end, it also accelerates spontaneous polymerization of actin from subunits. A detailed quantitative analysis of this effect using computer curve-fitting procedures has shown that capping protein accelerates spontaneous polymerization by promoting the formation of nuclei [24]. It has not yet been possible to distinguish among three different models that describe the intervention of capping protein at each of the three steps leading to the formation of a trimer. These models are almost equally effective in predicting the experimental data, but each model makes different predictions for the affinity of capping protein for actin monomers and dimers which will be tested experimentally in the future. Capping protein may specify the number of filaments in

the cell, because it is a powerful nucleating agent and there is enough capping protein, about one for every 100 actin monomers, for every filament in the cell to be capped at its barbed end. Since it binds to the barbed end of the filament, where many of the filaments are associated with the plasma membrane, and since it is localized in the cortex of the cell [23], we have speculated that capping protein is also involved with the association of filaments with the membrane.

Acanthamoeba has a protein that resembles α-actinin and cross links actin filaments, which we have called *GP-85* for gelation protein, with an 85,000 MW subunit [25]. Like α-actinin, it consists of two subunits in a rod-shaped molecule about 50 nm long. The *GP-85* binds weakly to actin filaments, with a dissociation constant >5 μM. Rheological measurements [26] of the physical properties of actin filament solutions show the the *GP-85* makes networks of actin filaments stiffer when the mechanical properties are evaluated at relatively high frequencies (0.1–1 cycle per sec) of deformation. In contrast, when the mechanical analysis is carried out at low frequencies, below 10^{-3} Hz, the actin filament samples have elastic and viscous moduli similar to actin filaments themselves. This interesting observation can be explained by the low affinity of *GP-85* for actin filaments. At low frequencies, the deformation of the network of filaments may occur at a rate slower than association-dissociation reaction of the cross links between the filaments. Consequently it appears that no cross links are present. In contrast, when the deformation occurs at high frequency, the cross links provide mechanical links between the filaments and stiffen the network. This behavior has important consequences for the physical properties of cytoplasm. A network of actin filaments cross linked by *GP-85* would resist rapid stresses such as saltatory movements of organelles, but were a stress applied slowly, as during cytokinesis, cross links between the filaments could rearrange rapidly enough for the filaments to slide past each other into new arrangements.

Acanthamoeba also contains a polypeptide that is similar in its size and immunological properties to spectrin from vertebrates [27]. The isolated molecule has a molecular weight of $\sim$ 260,000 and is about 100 nm long. Antibodies to this purified protein cross react with bona fide spectrin from vertebrate erythrocytes and brain, as well as high-molecular-weight proteins from every species that has been tested from insects to protozoa. The purified protein can also cross link actin filaments. In these ways, it appears to be similar to spectrin. On the other hand, it has been isolated without a second subunit polypetide chain that is characteristic of most spectrin molecules and does not appear to form the usual types of dimers and tetramers that are characteristic of spectrin. One possibility is that a second subunit has been lost during the purification procedure: An immunoreactive 240 kD polypeptide remains associated with the insoluble components of the cell during the initial extraction procedure. The *Acanthamoeba* spectrin is localized throughout the cytoplasm but is highly concentrated in the vicinity of the plasma membrane. In this way, it is also similar to spectrin from other cells. In that it is associated with plasma membrane and is capable of binding actin, it might provide part of the linkage between actin filaments and the plasma membrane that made it possible to isolate these two components as a complex many years ago [28].

CONCLUSIONS

It seems reasonable that this group of five proteins participates in an important way in the regulation of actin filament assembly and the organization of the actin

filaments in the living cell. It also seems reasonable that this is only a partial list of the actin-binding proteins in this cell and that further biochemical work will be necessary before we have a complete inventory. Of course, a complete inventory of these porteins will be necessary to understand, at the molecular level, how the system functions, because it is obvious already that these proteins form an interactive regulatory system inside the cell. To cite several examples: profilin inhibits nucleation whereas capping protein promotes nucleation; *GP-85* promotes actin filament cross linking, whereas capping protein inhibits this process, perhaps by shortening the actin filaments; and profilin and actophorin complement each other by sequestering actin monomers in nonpolymerizable complexes. The currently unidentified components of the system may serve equally important functions in the cell. Even this partial set of components is adequate to illustrate the points emphasized at the outset, including the importance of low-affinity interactions, the complexity of mechanisms, and the importance of mechanical analysis in the analysis of functions of cytoskeletal proteins.

Since none of these purified actin-binding proteins is regulated directly by Ca^{2+}, the common second messenger in cytoskeletal systems, it is worth considering the possibility that the assembly and organization of the actin filaments in *Acanthamoeba* are driven largely by mass action and not controlled in any very precise way. The low affinity of many of these interactions could very well be important in such a self-assembly process, providing enough flexibility because of weak interactions for the whole system to be rearranged when stressed by internal forces produced by myosin or by external forces. I speculate that much of the remodeling of the actin filament system, during cell movements such as locomotion or cytokinesis, is actually the simple consequence of localized activation of myosin leading to localized tension and deformation of a rather plastic system of actin filaments in the cell. This view suggests that the organization of actin filaments in the cell can be understood at least in part by simply learning how myosin is activated at specific times and places in the cytoplasm.

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