ATP-driven steady-state exchange of monomeric and filamentous actin from Dictyostelium discoideum

(nonmuscle cell motility/microfilaments/[35S]actin/nucleotide requirements/cytoskeleton)

PETER A. SIMPSON AND JAMES A. SPUDICH

Department of Structural Biology, Sherman Fairchild Center, Stanford University School of Medicine, Stanford, California 94305

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MATERIALS AND METHODS

Cells, Cell Growth, and Preparation of Actins. Unlabeled actin was obtained from D. discoideum, strain Ax-3, grown in HL-5 medium as described (11). Radiolabeled actin was isolated in the same way from amoeba grown in a chemically defined medium, FM of Franke and Kessin (12), modified to contain 0.5 mM rather than 2 mM L-methionine, and supplemented with 5.25 mCi of L-[35S]methionine per liter, at the highest specific radioactivity commercially available (980–1380 Ci/mmol, Amersham; 1 Ci = 3.7 × 10¹⁰ becquerels). Inocula for these cultures were from HL-5 medium, and growth in FM was for 3–5 days, or until the population had reached mid-logarithmic phase (1–2 × 10⁶ cells per ml). [35S]Actin prepared from these cells routinely had a specific radioactivity of 2–3 μCi/mg, and a 1-liter culture yielded about 15 mg of highly purified actin (greater than 95% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis; see ref. 11). An obvious advantage of preparing an actin probe by this in vitro labeling procedure is that the radiolabeled molecule contains no external modification that might alter its properties.

Preparation of Actin Monomers and Polymers. Monomeric actin was prepared as follows: F-actin from storage buffer (11) was collected by centrifugation at about 110,000 × g for 20 min in a Beckman Airfuge run at 23 pounds/inch² (160 kPa). Pellets were resuspended by sonication (Kontes Micro-Ultrasonic cell disrupter, setting 4) into G buffer (3 mM imidazole-HCl, pH 7.5/0.2 mM diithiothreitol/0.1 or 1.0 mM ATP). After incubation on ice for at least 1 hr, the solution of monomeric actin was clarified by a second centrifugation. Polymerization was initiated by the addition of KCl and MgCl₂ to give F buffer (G buffer containing 50 or 100 mM KCl and 1 mM MgCl₂).

Measurement of G- and F-Actin Pools by Sedimentation. The availability of [35S]actin allows for rapid analysis of microquantities of monomeric (G) and polymeric (F) actin by using a sedimentation assay. As will be shown elsewhere, centrifugation of a sample of [35S]actin, assembled to steady state, in siliconized Airfuge tubes for 15–20 min at 23 pounds/inch² is sufficient to separate the G- and F-actin pools. After such a centrifugation the concentration of actin in the supernatant as determined by liquid scintillation counting corresponds to the G(∞), or critical monomer concentration of actin. Values of G(∞) obtained by using the sedimentation assay agree well with

Abbreviations: G-actin, actin monomer; F-actin, actin filaments; G*-actin, [35S]actin monomer; F*-actin, [35S]actin filaments.

1 The word "exchange" is used in this paper to refer to the incorporation of actin monomer into filamentous actin at steady state. At steady state, the absolute concentrations of the G- and F-actin pools remain constant, and therefore, any incorporation of G-actin into F-actin must occur by an "exchange" reaction.
those obtained using the more conventional method of viscometric determination—i.e., 15–40 μg/ml (unpublished data).

All of the experiments described used the sedimentation assay to determine the distribution of label between G- and F-actins at steady state.

Preparation of the Equilibrium Dialysis Apparatus. The apparatus used was the model EMD 101 with 0.25 ml wells from Hoefer Scientific Instruments (San Francisco, CA). Millipore membranes (type DA, 13-mm diameter, 0.65-μm pore size) were pretreated for 30 min with bovine serum albumin solution at 0.5 mg/ml, then rinsed with F buffer to eliminate nonspecific binding of actin to the membranes. The rubber O-rings lining the wells of the dialysis apparatus were lubricated with silicone grease (Dow Corning) to prevent sample leakage. Each well contained a 3-mm-diameter glass bead to facilitate mixing, at a constant rate, during the course of the dialysis.

RESULTS

At Steady-State Equilibrium, G-Actin Rapidly Exchanges with F-Actin. To demonstrate a steady-state exchange reaction of the G- and F-actin pools, 35S-labeled G-actin was added in a trace amount to unlabeled F-actin, and the distribution of the radioactive label between the G- and F-actin pools was followed with time (Fig. 1). As soon as could be measured by the assay used (about 20 min), the added G*-actin was incorporated into F-actin to a level that remained stable for at least 27 hr. If the added G*-actin had remained monomeric, the result obtained would have been that shown by the broken line in Fig. 1.

The design of the experiment described above is less than ideal for two reasons. First, the addition of even a trace amount of labeled actin monomer leads to a change in the steady state concentration of monomer and filament. Under such conditions it is difficult to distinguish incorporation of G-actin into F-actin by exchange from that due to assembly that would necessarily occur to regain steady state. Second, there is a problem arising from the mechanical shearing of filaments that may be expected to occur upon mixing of the two actin populations. If the exchange were taking place by a mechanism that was depen-

dent on actin filament ends, such shear would lead to a change in the rate of the exchange reaction. We therefore wished to design an experimental approach whereby G*-actin could be introduced into a population of F-actin at steady state in such a way as to eliminate perturbation to this steady state, and which would not lead to a gross change in the physical structure of the population of F-actin.

An experimental situation that meets these criteria is presented in Fig. 2 Upper. The figure depicts an equilibrium dialysis chamber in which the two wells are separated by a membrane permeable to G-actin, but not to F-actin. On one side of this membrane, the H ( "hot") side, is placed a solution of [35S]actin assembled to steady state; on the other, the C ("cold") side, is placed an equivalent volume of F Buffer. In Fig. 2 Upper the chamber is set up for a control experiment to demonstrate that G*-actin, but not F*--actin, can cross the membrane from the H to the C side. As shown in Fig. 2 Lower, the amount of labeled actin that crosses the membrane increases with time and, as measured by the sedimentation assay, it is monomeric. In an experiment of this type the rate at which the

Fig. 1. Behavior of G*-actin added to F-actin at steady state. Unlabeled Dictyostelium G-actin at 500 μg/ml in 1.5 ml of G buffer containing 0.1 mM ATP was induced to assemble to steady state at 22°C by the addition of salts to give 100 mM KCl and 1 mM MgCl2. At time = 0, 0.02 ml of G*-actin was added to a final concentration of 10 μg/ml, with Vortex mixing. At intervals, samples were removed to determine the distribution of the labeled actin between the G- and F-actin pools by the sedimentation assay. The broken line represents the result expected if the added G*-actin were to remain monomeric. In this experiment the G(0), or critical monomer concentration, was 28 μg/ml.

Fig. 2. Equilibrium dialysis. (Upper) Schematic representation of chamber. (Lower) The membrane is permeable to G-actin but not to F-actin. Dictyostelium G*-actin at 100 μg/ml in 1.0 ml of G buffer containing 0.1 mM ATP was induced to assemble to steady state at 22°C by the addition of salts to give 100 mM KCl and 1 mM MgCl2. At time = 0, 200-μl samples of assembled actin solution and F buffer were pipetted into opposite wells of the dialysis apparatus and dialysis was initiated. At intervals, samples were removed from the C side of the chamber, and the distribution of labeled actin was determined by the sedimentation assay. Δ, Total cpm/150 μl of solution from the C side; ○, cpm/150 μl of supernatant (G*-actin); ●, cpm/corresponding pellet (F*-actin). The specific radioactivity of the [35S]actin used in this experiment was 4400 cpm/μg.
actin crosses the membrane is very slow, and the amount crossing within an hour represents only about 1–5% of the G*-actin. Because the actin on the C side of the membrane is present in an amount very much lower than the G(∞) concentration for this buffer, it is not expected to form filaments.

In other experiments, the dialysis chamber was set up with labeled actin on the H side of the membrane and an equivalent volume and concentration of unlabeled actin on the C side of the membrane. The actins were assembled to steady state under identical concentrations of concentration, buffer, temperature, and time. As dialysis was allowed to proceed, G*-actin crossed the membrane to the C side. Almost as rapidly as can be measured in the sedimentation assay, the G*-actin became incorporated into the F-actin pool (Fig. 3). This result is similar to that presented in Fig. 1, in that we observed a rapid exchange between the G- and F-actin pools.

It is important to note that in this situation actin monomer is free to travel in both directions across the membrane, and that for every molecule of actin that crosses from the H to the C side of the membrane, one passes in the opposite direction. As a result, although there is no overall change in the concentration of G- or F-actin on either side of the membrane, a trace amount of G*-actin is added to the population of unlabeled actin. In addition, because mixing is gentle and constant in both populations of actin, there is no possibility of selective shearing of actin filaments leading to anomalous results.

We conclude that, at steady state, there is a rapid exchange of actin subunits between the G-actin pool and the F-actin pool.

The Exchange Reaction Requires ATP. ATP is not necessary for the KC1- or MgCl2-induced formation of actin filaments from G-actin (13–15). However, as shown in Fig. 3, the exchange reaction between G-actin and F-actin requires the presence of ATP. Given that G-actin in the absence of ATP is very unstable (see ref. 14), one might argue that this observed lack of exchange of ADP-G-actin into F-actin is the result of actin denaturation. However, it has been shown that the 3 mM concentration of ATP used in these experiments is sufficient to stabilize G-actin in the absence of ATP (16). Furthermore, we have shown by two types of experiments that the ADP-G*-actin on the C side of the dialysis membrane is indeed capable of assembly into filaments. At time = 2 hr in an experiment such as that shown in Fig. 3, 2 mM ATP was added to the C side; 70% of the G*-actin became sedimentable within 1 hr.

In the second type of experiment, the ADP-G*-actin removed from the C side at time = 2 hr was mixed with unlabeled ADP-G-actin at 1.0 mg/ml and copolymerized in the absence of ATP; 35% of the ADP-G*-actin became sedimentable within an hour.

The Exchange Reaction Requires Filament Ends. The non-steady-state assembly of actin filaments is markedly inhibited by submicromolar concentrations of cytochalasin D, apparently by interaction with the end of the filaments. The rate of exchange in filament assembly (16–20, §). Thus, if the exchange reaction at steady state involves primarily the ends of the filaments, submicromolar amounts of cytochalasin should inhibit the exchange. If however, the exchange occurs primarily along the length of the filaments, this amount of cytochalasin should not be expected to inhibit the exchange. As shown in Fig. 4, cytochalasin D (Sigma) at 0.1 μM (1 molecule of cytochalasin

\[ \text{FIG. 3. The exchange reaction requires ATP. For the ATP experiments (C), labeled (3000–6000 cpmpg) and unlabeled Dictyostelium actins were at 562 μg/ml in G buffer containing 1.0 mM ATP. For the ADP experiments (C), labeled and unlabeled Dictyostelium actins were at 1000 μg/ml in G buffer containing 3.0 mM ADP. In all cases, assembly of filaments to steady state at 22°C was induced by the addition of salts to give 50 mM KCl and 1 mM MgCl2. To ensure that no ATP was present in the ADP experiments, G-actins were prepared from storage buffer in solutions containing 3.0 mM ADP. The high concentration of actin in the ADP experiments was necessary to offset the increased G(∞) seen in ADP, approximately 300 μg/ml, compared to 20–30 μg/ml in ATP. As time = 0, 0.000–1.1 samples of the labeled and unlabeled actin solutions were pipetted into opposite wells of the dialysis apparatus and dialysis was initiated. At intervals, samples were removed from the C side of the chamber, and the distribution of labeled actin was determined by the sedimentation assay. The ATP curve is a composite of the data from seven experiments; the ADP curve is a composite of the data from four experiments.}

\[ D \text{ per 50 actin monomers} \text{ decreases the rate of exchange to less than 1/10th of the control value.}

\[ \text{DISCUSSION}

We have demonstrated that the pools of monomeric and filamentous actin that coexist at steady state undergo a rapid exchange reaction \textit{in vitro} that is dependent on the presence of ATP and that involves primarily actin filament ends.

The role of ATP in the assembly of actin monomers into filaments remains unclear (see ref. 21), especially because assembly can occur in ADP alone (13) or in the complete absence of nucleotide (14, 15). Although adenine nucleotides and divalent cations have been suggested as possible regulators of the rate and extent of actin polymerization \textit{in vitro} (14), a change from ATP to ADP increases the time necessary for complete assembly only 3 to 5-fold (22). The striking effect of replacing ATP by ADP on the rate of the G-F-actin exchange reaction (Fig. 3) suggests that, \textit{in vitro}, the role of ATP in the actin system may primarily involve the steady-state exchange reaction

\[ s \text{ S. Brown and J. A. Spudich, unpublished.} \]

\[ s \text{ In all of the experiments in this report there is a small amount (0.5–5%) of nonpolymerizable actin in the actin preparations. In the dialysis experiments this nonpolymerizable G*-actin accumulates on the C side of the membrane while the polymerizable G*-actin is trapped in the unlabeled F-actin. This phenomenon will be dealt with quantitatively in another report.} \]

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and not the assembly or polymerization reaction. Thus an absolute requirement has hereby been demonstrated for ATP in an actin system.

Cytochalasin D at submicromolar concentrations strongly inhibits the exchange reaction between G-actin and F-actin (Fig. 4). Given the reported effects of the cytochalasins on actin filament assembly (16–20), it is likely that cytochalasin D is inhibiting the exchange reaction by interfering with the ends of the actin filaments. The cytochalasin D inhibition of the rate of exchange is not complete and, as yet, it is not possible to discern whether this slow residual exchange is due to events at filament ends or to events along the length of the filament, an important distinction when one considers mechanisms for the exchange reaction.

One possible steady-state exchange mechanism that is consistent with the demonstrated requirements for ATP and filament ends is the head-to-tail model for actin filament assembly recently proposed by Wegner (10). In this elegant model, one end of the polar (22) actin filament is primarily an assembly end; here monomer–polymer association events outnumber dissociation events. The other end of the filament is primarily a disassembly end. At steady state, net associations and dissociations of monomer at the respective ends of the filament result in a treadmilling of actin through the filament. As pointed out by Wegner on theoretical grounds (10), if actin filaments behave as treadmills at steady state, there must be energy available to drive the reaction, presumably from the hydrolysis of ATP.

The mechanism of the exchange reaction could be considerably more complex than a simple treadmill model, and in order to understand the details of the reaction more data are necessary. The important point for this paper is that we have demonstrated that there is a rapid exchange reaction between G-actin and F-actin at steady state, and, more importantly, that this reaction is dependent on the presence of ATP.

The ATP-driven exchange between G-actin and F-actin could be critically important in the ever-changing temporal and spatial organization of actin filaments within the cell. Filaments may be in a constant flux, and a site in need of filaments, such as the future furrow region of a dividing cell, may recruit actin from other filaments merely by establishing nucleation sites for filament assembly. Thus, there may be a constant, general flow of actin monomers from one part of the cell to filaments in other areas via a G-actin pool that is not necessarily larger than the G(∞) concentration. In addition, accessory proteins or small molecules may regulate the rates of assembly and disassembly of actin filaments within the cell by interacting with filament ends or with actin monomers.

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