Actin Filaments Undergo Limited Subunit Exchange in Physiological Salt Conditions

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ABSTRACT The exchange of actin filament subunits for unpolymerized actin or for subunits in other filaments has been quantitated by three experimental techniques: fluorescence energy transfer, incorporation of 35S-labeled actin monomers into unlabeled actin filaments, and exchange of [14C]ATP with filament-bound ADP. In the fluorescence energy transfer experiments, actin labeled with 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid (IAENS) served as the fluorescent energy donor, and actin labeled with either fluorescein-5-isothiocyanate (FITC) or fluorescein-5-maleimide (FM) served as the energy acceptor. Fluorescent-labeled actins from Dictyostelium amoebae and rabbit skeletal muscle were very similar to their unlabeled counterparts with respect to critical actin concentration for filament assembly, assembly rate, ATP hydrolysis upon assembly, and steady-state ATPase. As evidenced by two different types of fluorescence energy transfer experiments, <5% of the actin filament subunits exchanged under a variety of buffer conditions at actin concentrations >0.5 mg/ml. At all actin concentrations limited exchange to a plateau level occurred with a half-time of about 20 min. Nearly identical results were obtained when exchange was quantitated by incorporation of 35S-labeled Dictyostelium actin monomers into unlabeled muscle actin or Dictyostelium actin filaments. Furthermore, the proportion of filament-bound ADP which exchanged with [14C]-ATP was nearly the same as actin subunit exchange measured by fluorescence energy transfer and 35S-labeled actin incorporation. These experiments demonstrate that under approximately physiologic ionic conditions only a small percentage of subunits in highly purified skeletal muscle or Dictyostelium F-actin participate in exchange.

Steady-state exchange of G-ATP-actin with subunits of F-ADP-actin was first postulated in 1960 by Asakura and Oosawa (1, 2). They discovered that polymerization of actin was accompanied by an initial rapid burst of phosphate liberation followed by slower ATP hydrolysis of indefinite duration. Because the initial hydrolysis of 1 mol ATP per mol actin was known to accompany assembly of actin monomers into filaments, they postulated that further steady-state ATP hydrolysis represented continual incorporation of G-ATP-actin into filaments followed by ATP hydrolysis and disassembly of G-ADP subunits from the filament (1). Previous work had established that [14C]ATP exchanges rapidly with G-ADP-actin and appears as tightly bound ADP in F-actin (3). Therefore, if steady-state actin exchange involved all of the subunits in the filament, exchange would result in the incorporation into filaments of 1 mol 14C-nucleotide per mol of F-actin subunits. However, in [14C]ATP experiments by Gergely et al. (4) an average of only 11% nucleotide exchange was observed in filaments polymerized in 0.7 mM MgCl2 and maintained at steady-state equilibrium for up to 24 h.

In 1976, the exchange proposal of Asakura and Oosawa (1) was extended by Wegner (5), who theorized that steady-state ATP hydrolysis, when coupled to the known polarity of actin filaments (6), could result in net incorporation of G-ATP-actin at one end of the filament with corresponding depolymerization of G-ADP-actin from the opposite filament end. Such head-to-tail polymerization at steady-state equilibrium would result in complete exchange of filament subunits for free monomers with each turn of the filament “treadmill.” Accordingly, Wegner (5, 7) was able to demonstrate nearly complete exchange of actin monomers into filaments within 3-4 h when actin was preassembled to steady-state equilibrium in the absence of KCl, in low levels of MgCl2 (0.5 mM), or in 1.2 mM CaCl2.

Since intracellular concentrations of K+ are generally believed to be in the 100 mM range (8, 9), and Mg2+ and ATP concentrations of 1-5 mM are expected (10, 11), we wished to
know the extent of filament subunit exchange under salt conditions presumably appropriate to the cytoplasm. Therefore, we have directly quantitated actin filament subunit exchange using two independent methods, 35S-labeled actin monomer exchange into filaments (12) and fluorescence energy transfer between neighboring actin subunits within a filament (13, 14). Furthermore, [14C]ATP incorporation into highly purified F-actin was used to correlate subunit exchange with nucleotide exchange.

MATERIALS AND METHODS

Buffers

Buffer A: 2 mM TRIS, pH 8.0 at 25°C, 0.2 mM Na2ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl2, 0.005% NaN3.

IAENS, FM labeling buffer: 10 mM K3HPO4, pH 8.0 at 4°C, 0.2 mM Na2ATP, 0.000% NaN3, 0.1 M KCl, 1 mM MgCl2.

FITC labeling buffer: 50 mM K2CO3, pH 9.3 at 4°C.

TES G-buffer: 2 mM TES, pH 7.2 at 25°C, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 50 mM CaCl2, 0.005% NaN3 (contains ~2 mM K+ added as KOH to titrate 2 mM TES to pH 7.2). Note: In some experiments 20 μM MgCl2 was substituted for 50 mM CaCl2. Substitutions are noted in the text.

Imidazole-G-buffer: 3 mM imidazole, pH 7.5 at 25°C, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.005% NaN3.

Pipes buffer: 10 mM Pipes, pH 7.0 at 25°C, 0.5 mM ATP, 0.5 mM dithiothreitol, 2.5 mM MgCl2 (contains ~10 mM K+ added as KOH to titrate 10 mM Pipes to pH 7.0).

Actin Purification

Rabbit skeletal muscle actin isolated by the method of Spudich and Watt (15), was recycled by sedimentation of F-actin, homogenization of pelleted actin into Buffer A, depolymerization by dialysis for 4-5 h against Buffer A at 4°C, clarification at 150,000 g for 1.5 h, and repolymerization of the clarified supernatant G-actin with 0.1 M KCl, 1 mM MgCl2, and 1 mM ATP (16).

Recycled actin was further treated either with DEAE cellulose (Whatman DE-52) to remove trace contaminants (16) or by gel filtration through G-150 Sephadex in Buffer A to obtain actin monomers (17). In many experiments, actin was treated by both DEAE and gel filtration. Monomers were polymerized by addition of KCl to 0.1 M and MgCl2 to 1 mM and stored on ice. Purified actin was >99% homogeneous (16) by SDS PAGE at a protein loading of 10-20 μg on a 1.5-mm slab gel containing 10% acrylamide (18). Final recoveries of actin were 40-50% after DEAE treatment and 30-40% after DEAE, depolymerization, and gel filtration.

Dictyostelium discoidum actin was purified by the method of Uyemura, et al. (19). Isolated F-actin was further purified by the following recycle method: the F-actin was sedimented at 150,000 g for 1 h at 4°C, depolymerized by dialysis for 4-6 h against Buffer A, clarified at 150,000 g for 1.5 h, and reassembled by addition of 0.1 M KCl and 1 mM MgCl2.

Dictyostelium actin was obtained which contained 0.8-1.0 moi FITC per mol actin. Lower values.

Properties of Fluorescent-labeled Actins

ACTIN RECYCLING: Fluorescent-labeled actins were found to be less stable to long exposure in G-actin buffers than unlabeled actin. Overnight dialysis in G-actin buffers at 4°C resulted in high critical actin concentrations (150-200 μg/ml) upon reassembly under optimal assembly conditions (0.1 M KCl, 2 mM MgCl2). Consequently, all actins were quickly recycled in the experimental buffer of choice immediately before use. F-actin was sedimented at 150,000 g for 1 h at 4°C. The pelleted actin was homogenized gently into the experimental G-actin buffer of choice to a concentration of ~2 mg/ml and dialyzed in collodion bags for 2 h against 1 liter of G-buffer. The depolymerized actin was clarified by high-speed centrifugation (150,000 g, 1 h, 4°C) and the G-actin was immediately used in the experiments. Both fluorescent-labeled and unlabeled actins depolymerized rapidly when dialyzed in collodion bags against G-actin buffers, giving complete depolymerization within 2 h at actin concentrations of <2 mg/ml. Fluorescent-labeled actin recycled just before use gave the critical concentrations reported in Table 1, and no increase in critical actin concentration or decrease in viscosity occurred in the time course of the exchange experiment.

CRITICAL ACTIN CONCENTRATION: Recycled IAENS-, FM-, and FITC-labeled G-actins at 0.25 and 1 mg/ml in TES G-buffer were polymerized separately by addition of KCl to 0.1 M and MgCl2 to 1 mM followed by incubation for 2-3 h at 25°C. In a separate experiment 0.2 mg/ml FITC-labeled actin was copolymerized with 0.05 mg/ml IAENS-labeled actin. Polymerized samples (100 μl) were sedimented for 1 h at 150,000 g in an anfuge (Beckman Instruments, Palo Alto, CA), the supernatant fraction was decanted, and 20-μl aliquots were assayed for protein concentration with 2 ml of Bradford reagent (21) at 595 nm. Critical actin concentration (Ca) was determined by comparison to a standard curve generated from known concentrations of IAENS- or FITC-labeled G-actins. Results of critical concentration determinations are given in Table 1.

TABLE I

Assembly Properties of Fluorescent-Labeled Actin

| Ca (mg/ml) | Mol ATP hydrolyzed per mol actin during assembly | Mol ATP hydrolyzed per mol actin per h at steady-state |
|------------|-----------------------------------------------|------------------------------------------------------|
| Unlabeled  | 20 | 1.5 | 0.9 | 0.03 |
| IAENS-labeled | 30 | 1.2 | 1.0 | 0.04 |
| FITC-labeled | 40 | 1.5 | 0.9 | 0.07 |
| FM-labeled  | 30 | 1.5 | — | — |
| IAENS-, FITC-coassembled | 30 | 1.2 | 0.9 | 0.03 |

* Actin at 0.25 mg/ml in TES G-buffer was assembled by addition of 0.1 M KCl and 1 mM MgCl2. Ca determinations at 1 mg/ml gave nearly identical values.

PARDEE ET AL. Limited Subunit Exchange in Actin Filaments 317
 Assembly by Fluorescence Energy Transfer

Immediately before each exchange experiment, donor and acceptor labeled G-actins were coassembled to determine the maximum percent quench and critical actin concentration under the conditions of the exchange assay. In a typical experiment, FITC-labeled G-actin and IAENS-labeled G-actin in TES G-buffer were mixed in a 4:1 FITC-labeled actin:IAENS-labeled actin ratio to a final concentration of 1.5 mg/ml in 0.3 M KCl. The baseline fluorescence of the labeled G-actin mixture was determined at 470 nm for several minutes. The labeled G-actin mixture was then assembled by addition of 30 µl of a 10-times concentrated KCl, MgCl₂ stock solution to achieve final concentrations of 0.1 M KCl and 1 mM MgCl₂. Assembly was monitored by the decrease in fluorescence at 470 nm resulting from energy transfer between IAENS- and FITC-labeled subunits. After assembly was complete, as evidenced by no further fluorescence changes, the sample was placed on ice to freeze. In a 30 s to 1 min cuvette, sonicated on ice for 30 s to 1 min with a Kontes micro-ultrasonic cell disruptor (Kontes Glass Co., Vineland, NJ) fitted with a 4.5 inch probe at a power setting of 8 (9.9 W, 25 KHz), and returned to the fluorometer to determine the effect of sonication on the fluorescence signal of coassembled filaments. Subunit Exchange Quantitations

DETERMINATION OF FILAMENT SUBUNIT EXCHANGE BY FLUORESCENCE ENERGY TRANSFER: Two different types of fluorescence energy transfer experiments were used to quantify actin filament subunit exchange. In the first method, unlabeled actin filaments were mixed with coassembled filaments containing donor (IAENS) and acceptor (FITC)-labeled actin subunits, and the mixed filament population was monitored at 470 nm for increases in fluorescence (relief of quench). Filaments were mixed by several gentle passes through a 9-inch Pasteur pipette. Complete mixing was evidenced by a decrease in signal at 470 nm which corresponded exactly to the predicted dilution of IAENS- and FITC-labeled coassembled filaments after mixing with unlabeled filaments. The fluorescence at 470 nm was monitored at 1 h intervals for up to 6 h at 25°C. Samples remained in the fluorometer at 25°C with excitation and emission light shutters closed during the intervals between monitoring. The percent subunit exchange was calculated from the relationship:

\[
\text{Percent subunit exchange} = \frac{F_{\text{final}} - F_{\text{initial}}}{F_{\text{initial}} - F_{\text{sonicated}}} \times 100
\]

where \(F_{\text{final}}\) = fluorescence of the filament mixture immediately after mixing, \(F_{\text{initial}}\) = fluorescence of the filament mixture before the exchange period, and \(F_{\text{sonicated}}\) = fluorescence of the filament mixture after sonication.

In the second method for quantitating subunit exchange, filaments labeled only with fluorescence donor (IAENS) were mixed with an excess of filaments labeled only with acceptor (either FITC or FM). In this system, exchange results in a decrease in donor-labeled actin fluorescence at 470 nm (increased quench). For example, for measurements of exchange at 1 mg/ml total actin, IAENS-labeled G-actin (1 mg/ml) and FM-labeled G-actin (2 mg/ml) were assembled separately by addition of KCl to 0.1 M and MgCl₂ to 1 mM and incubated for 2 h at 25°C in the dark. A 1:4 ratio of IAENS-labeled F-actin to FM-labeled F-actin was then gently mixed by five passes through a 9-inch Pasteur pipette and immediately monitored at 470 nm for changes in fluorescence (increased quench). Final concentrations were 0.2 mg/ml IAENS-labeled F-actin and 0.8 mg/ml FM-labeled F-actin. Fluorescence of the filament mixture at 470 nm was monitored either continuously for 1-2 h or at 1-h intervals for up to 6 h at 25°C. Samples remained in the fluorometer at 25°C with light shutters closed between readings. When no further changes in fluorescence occurred, the filament mixture was removed from the cuvette, sonicated for 30 s on ice, and the fluorescence at 470 nm retested for several minutes. Samples were resonicated with 30-s bursts until no further increases in quench were observed (total randomization of subunits, 100% exchange). Percent subunit exchange was calculated from the fluorescence observed immediately after mixing donor and acceptor-labeled filaments (\(F_{\text{initial}}\)) at the end of the exchange period (\(F_{\text{final}}\)), and after sonication (\(F_{\text{sonicated}}\)).

\[
\text{Percent subunit exchange} = \frac{F_{\text{initial}} - F_{\text{final}}}{F_{\text{sonicated}} - F_{\text{initial}}} \times 100
\]

As an internal check on the accuracy of the exchange quantitation, the maximum percent quench obtained after sonication was compared to the percent quench obtained for coassembly of separate aliquots of IAENS-labeled G-actin (0.2 mg/ml) and FM-labeled G-actin (0.8 mg/ml). Agreement of these values to within 1-2% ensured accurate quantitation of filament subunit exchange. By this procedure, exchange was quantitated for different concentrations of fluorescent-labeled actins prepared in various G-buffers and assembled under several K⁺, Mg²⁺, and ATP concentrations. Specific buffer conditions, actin concentrations, and actin sources are given in the figure legends.
INCORPORATION OF \(^{35}\text{S}\)-LABELED ACTIN MONOMER INTO FILAMENTS: \(^{35}\text{S}\)-labeled Dictyostelium F-actin and unlabeled F-actin from either rabbit muscle or Dictyostelium were separately recycled immediately before each exchange experiment. F-actin was sedimented at 150,000 g for 1 h at 25°C in an airfuge. Pelleted actin was homogenized into imidazole G-buffer, dialyzed for 2–3 h at 4°C in 10,000-dalton cutoff collodion bags to depolymerize the actin, and clarified by centrifugation at 150,000 g for 1 h at 4°C. Unlabeled muscle or Dictyostelium G-actin (1 ml, 1 mg/ml) was assembled for 2 h by addition of KCl to 0.1 M, MgCl\(_2\) to 1 mM and ATP to 1 mM. The resulting F-actin was then gently mixed with 2 \(\mu\)l of 0.25 mg/ml \(^{35}\text{S}\)-labeled G-actin (sp act = 3,000 cpm/\(\mu\)g) by inverting the mixture several times in a conical 1.5 ml microfuge tube. Exchange assay samples were incubated at 25°C for 3 h at which time F-actin was sedimented by centrifugation for 1.5 h at 150,000 g, 25°C. Pelleted actin was rinsed with imidazole G-buffer containing 0.1 M KCl and 1 mM MgCl\(_2\), sonicated into 0.5 ml H\(_2\)O, dispersed in 10 ml of ACS scintillant (Amerham, Arlington Hills, IL), and counted. Percent exchange of F-actin subunits with \(^{35}\text{S}\)-labeled actin monomer was calculated from the distribution of \(^{35}\text{S}\)-labeled actin specific activity between sedimentable (F) and nonsedimentable actin (CA):

\[
\text{Percent exchange} = \frac{\text{cpm in pellet/F-actin}}{\text{cpm in supernatant/F-actin}} \times 100
\]

\[
= \frac{\text{Sp. act. of F-actin}}{\text{Sp. act. of CA}} \times 100
\]

The concentration of actin remaining in the 150,00 g supernatant was determined on three 20-\(\mu\)l aliquots by Bradford assay (21).

To determine the assembly competence of \(^{35}\text{S}\)-labeled actin remaining in the supernatant fraction after complete exchange, 100 \(\mu\)l of supernatant fraction was mixed with 200 \(\mu\)l of 1 mg/ml unlabeled muscle G-actin, and the mixture was assembled by addition of KCl to 0.1 M and MgCl\(_2\) to 1 mM. After assembly for 2 h at 25°C the F-actin was sedimented at 150,000 g for 1 h at 25°C in an airfuge. Pelleted actin was homogenized into TES G-buffer to a concentration of 5 mg/ml. This actin was recycled by depolymerization in TES G-buffer, clarified at 150,000 g for 1.5 h at 25°C, and repolymerized by addition of KCl to 0.1 M and MgCl\(_2\) to 1 mM. [\(^1\text{H}\)G-actin (sp act = 3,000 cpm/\(\mu\)mol) (Amerham, Arlington Hills, IL) was added to the F-actin to give 1,500 cpm/\(\mu\)l of sample. Samples were mixed by five passages through a 9-inch Pasteur pipette and the mixture incubated at 25°C.

Total sample nucleotide concentration and actin concentration were determined by UV spectra. To separate F-actin from nonsedimentable actin and unbound [\(^{1}\text{H}\)G-actin at various times after mixing, 50-\(\mu\)l aliquots of the incubation mix were gently layered over 50 \(\mu\)l of 20% sucrose in an airfuge tube and centrifuged for 20 min at 150,000 g at 25°C. Sucrose retarded the diffusion of unbound [\(^{1}\text{H}\)G-actin into the F-actin pellet during sedimentation. The supernatant fraction was removed and the F-actin pellet gently rinsed with 20% sucrose. Pellets were sonicated into 100 \(\mu\)l of H\(_2\)O and counted. Control experiments in which 5 \(\mu\)l of [\(^{1}\text{H}\)G-actin were placed onto the F-actin which had been overlayed on the sucrose cushion demonstrated that 99% of the free [\(^{1}\text{H}\)G-actin could be separated from the pelleted F-actin by centrifugation. Therefore, control values of 800 cpm were subtracted from the cpm incorporated into the pelletable actin. The percentage of F-actin which had incorporated ATP during the time course of the experiment was calculated by:

\[
= \frac{\text{cpm actin/cpm total ATPcin}}{\text{Actin added}} \times 100
\]

The critical actin concentration in this experiment was 20 \(\mu\)g/ml (0.4% of the actin present); control experiments demonstrated that >95% of the F-actin was sedimented.

RESULTS

Characterization of Fluorescent-labeled Actin

Dye-labeled actins were characterized with respect to their individual fluorescent properties. IAENS-labeled actin exhibits a broad fluorescence emission spectrum with a maximum at 470 nm (Fig. 1 A) compared with an emission spectrum sharply peaked at 520 nm for FITC-actin (Fig. 1 B). The emission and absorption properties of FM-labeled actin are very similar to those of FITC-labeled actin (data not shown). FITC and IAENS emission spectra are well resolved with no FITC fluorescence observed at the 470 nm fluorescence maximum of IAENS-labeled actin. When IAENS-labeled G-actin or FITC-labeled G-actin are individually assembled into filaments, assembly-dependent changes in the emission spectra occur. The IAENS-labeled G-actin spectrum is shifted slightly toward shorter wavelengths and a 11% increase in the emission intensity at 470 nm (Fig. 1 B). Assembly of a mixture of IAENS-labeled G-actin and FITC-labeled G-actin resulted in 55–60% quench of IAENS-labeled actin fluorescence at 470 nm when the mole fraction of FITC-labeled actin in the filaments was 0.65–0.70.
from an IAENS donor on one actin subunit to a FITC acceptor on a neighboring actin subunit in the assembling filament (13, 14). Fluorescent-labeled actin subunits must be \( \leq 70 \) Å apart to allow energy transfer from IAENS-labeled units to FITC- or FM-labeled units (13, 14). For this energy transfer system, in which IAENS-labeled actin acts as the fluorescent donor and FITC-labeled actin (or FM-labeled actin) as the acceptor, 55–60% of the IAENS fluorescence at 470 nm is transferred (quenched) during actin assembly when the mole fraction of FITC in filaments is 0.65–0.70.

The degree of fluorescence quench of donor-labeled actin is proportional to the mole fraction of acceptor-labeled actin within the coassembled filament. The quench curve resulting from coassembly of IAENS-labeled G-actin with increasing proportions of FITC-labeled G-actin is shown in Fig. 2 (open circles). An identical quench curve is observed if the mole fraction of FITC-labeled actin within the coassembled filaments is obtained by sonicating coassembled filaments in the presence of increasing concentrations of unlabeled F-actin (Fig. 2, closed circles). We conclude that sonication under these conditions leads to complete randomization of unlabeled and fluorescent-labeled monomers among actin filaments, but sonication does not otherwise alter the energy transfer properties of fluorescent-labeled actin filaments.

The percent quench of IAENS-labeled actin within the filament is independent of F-actin concentration (Fig. 3). Thus, interfilament energy transfer is not appreciable at the actin concentrations used in this study. Furthermore, the quench is independent of the total filament concentration at a particular mole fraction of energy acceptor. Full assembly is equivalent to \( \sim 55\% \) quench at all F-actin concentrations used when the mole fraction of FITC-labeled actin is 0.60–0.65.

**Quantitation of Filament Subunit Exchange**

**Exchange between Coassembled IAENS-, FITC-labeled Actin Filaments, and Unlabeled Filaments.** We have used several experimental approaches to determine the extent of subunit exchange among filaments at steady-state in the presence of physiologically related salt conditions. Two different types of fluorescence energy transfer experiments were used. In the first experiment coassembled donor-, acceptor-labeled filaments were mixed with unlabeled filaments, and the amount of exchange was quantitated by the decrease in fluorescence quench. As shown in Fig. 4, a maximum of 55% quench was obtained for fully assembled filaments containing a mole fraction of FITC-labeled actin of 0.68. At all concentrations of actin tested (0.05–2 mg/ml), a hyperbolic assembly curve was observed with assembly half-times ranging from 40 min at 0.05 mg/ml to 1 min at 2 mg/ml for assembly with 0.1 M KCl + 1 mM MgCl2. Sonication of the coassembled filaments did not alter the observed quench since the filament subunits were already randomized. The critical actin concentrations for assembly of these donor-, acceptor-labeled filaments were similar to those for unlabeled filaments (Table I).

To measure exchange, unlabeled muscle actin filaments were added to the coassembled muscle actin filaments. No change in quench was detected for 6 h at 25°C (Fig. 5). The sample was subsequently sonicated, causing complete randomization of the fluorescent-labeled actin among filaments. Sonication caused a decrease to a quench value of 15%, the expected value for completely randomized filaments with an FITC-labeled actin mole fraction of 0.14 (Fig. 2). We conclude that <5% of the filament subunits exchanged before sonication. The same result (<5% exchange) was obtained for exchange between coassembled muscle actin and unlabeled Dictyostelium actin.

**Exchange between IAENS-labeled Actin Filaments and FITC- or FM-labeled Actin Filaments:**

The second method of determining interfilament subunit exchange by fluorescence energy transfer was to mix populations of either muscle or Dictyostelium IAENS-labeled F-actin with FITC- or FM-labeled F-actin and monitor the resulting in-

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**Figure 2:** Efficiency of energy transfer between IAENS-labeled actin and FITC-labeled actin. Mixtures of IAENS-labeled, FITC-labeled, and unlabeled muscle G-actin were coassembled by addition of KCl to 0.1 M and MgCl2 to 1 mM. Five-micromolar mixtures of FITC-labeled + unlabeled actin containing an increasing proportion of FITC-labeled actin were used to determine the extent of subunit exchange among filaments at steady-state in the presence of physiologically related salt conditions. Two different types of fluorescence energy transfer experiments were used. In the first experiment coassembled donor-, acceptor-labeled filaments were mixed with unlabeled filaments, and the amount of exchange was quantitated by the decrease in fluorescence quench. As shown in Fig. 4, a maximum of 55% quench was obtained for fully assembled filaments containing a mole fraction of FITC-labeled actin of 0.68. At all concentrations of actin tested (0.05–2 mg/ml), a hyperbolic assembly curve was observed with assembly half-times ranging from 40 min at 0.05 mg/ml to 1 min at 2 mg/ml for assembly with 0.1 M KCl + 1 mM MgCl2. Sonication of the coassembled filaments did not alter the observed quench since the filament subunits were already randomized. The critical actin concentrations for assembly of these donor-, acceptor-labeled filaments were similar to those for unlabeled filaments (Table I).

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**Figure 3:** Effect of filament concentration on fluorescence quench. Muscle FITC-labeled G-actin and IAENS-labeled G-actin in TES G-buffer were mixed to obtain final total actin concentrations of 0.1 to 1 mg/ml. The mole fraction of acceptor (FITC-labeled actin) was 0.60–0.65 in all samples. Mixtures were assembled at 25°C by addition of KCl to 0.1 M and MgCl2 to 1 mM. Critical actin concentrations (CA) of 30 µg/ml were subtracted from the total actin concentration to obtain the concentration of F-actin. Total observed quench was also corrected for the measured CA.
increase in donor fluorescence quench. In this type of experiment, percent exchange is directly proportional to the percent increase in quench. When FITC acceptor-labeled filaments were mixed into donor-labeled filaments, <2% increase in quench was observed which was completely terminated within 60 min (Fig. 4). Sonication resulted in 56% quench which was in close agreement with the maximum quench obtained by coassembly of donor-G-actin with FITC acceptor-G-actin (55% quench; Fig. 4). Therefore at a concentration of 1 mg/ml F-actin, subunit exchange is <5%. The experiments were repeated using FM-labeled F-actin at an FM-labeled actin mole fraction of 0.65. The fluorescence emission was monitored at 470 nm. After a 10 min incubation at 25°C to establish the baseline, actin was assembled by addition of KCl to 0.1 M, MgCl₂ to 1 mM, and ATP to 1 mM. After assembly, the sample was sonicated. No further increase in quench was observed, indicating that coassembly had resulted in complete randomization of the actin units. It is also evident that sonication per se does not alter the fluorescence intensity.

**Figure 4** Detection of actin assembly by fluorescence energy transfer. Rabbit skeletal muscle IAENS-labeled G-actin (0.2 mg/ml final concentration) in TES G-buffer was mixed with FITC-labeled G-actin (0.8 mg/ml final concentration, mole fraction FITC-labeled actin, 0.65). The fluorescence emission was monitored at 470 nm. After a 10 min incubation at 25°C to establish the baseline, actin was assembled by addition of KCl to 0.1 M, MgCl₂ to 1 mM, and ATP to 1 mM. After assembly, the sample was sonicated. No further increase in quench was observed, indicating that coassembly had resulted in complete randomization of the actin units. It is also evident that sonication per se does not alter the fluorescence intensity.

**Figure 5** Subunit exchange between fluorescent-labeled filaments and unlabeled filaments. Muscle IAENS-labeled G-actin (0.2 mg/ml) and FITC-labeled G-actin (1.2 mg/ml) in TES G-buffer were coassembled with 0.1 M KCl + 1 mM MgCl₂ + 1 mM ATP. Coassembled filaments containing a mole fraction FITC-labeled actin of 0.65 were gently mixed with unlabeled filaments. Final concentrations were 0.2 mg/ml coassembled filaments and 1.0 mg/ml unlabeled filaments. The fluorescence intensity of the mixture was monitored intermittently at 470 nm. No fluorescence change was detected after 6 h at 25°C. The mixture was then sonicated, which randomized the subunits and thereby decreased the quench to 15%, the value expected for randomized filament subunits containing a mole fraction of FITC of 0.13 (see Fig. 2). The same result (<5% exchange) was obtained for experiments in which coassembled muscle F-actin labeled with IAENS and FM were mixed with unlabeled Dicytostelium F-actin. In this case, 35% maximum quench was observed for coassembled IAENS, FM filaments.

**Exchange of 35S-labeled actin monomers from Dicytostelium into preexisting unlabeled actin filaments** (12). This method has the advantage that actin is not covalently modified by addition of a probe, as in the fluorescence experiments. As previously demonstrated (12), 35S-labeled actin monomers are quickly incorporated into filaments up to a plateau level of ~50-70% of the added monomer. The half-time of incorporation is approximately 15 min with no further incorporation after 30-40 min (12). The amounts of exchange of 0.5, 1.0, and 10 µg/ml 35S-labeled G-actin into 1 mg/ml unlabeled muscle F-actin are presented in Table II. The plateau levels of exchange were <5%. Assays for assembly competency of the 35S-labeled actin after the 3-h incubation in the exchange assays (see Materials and Methods) demonstrated that >90% of the unexchanged 35S-labeled actin was still capable of assembly. Percent exchange was independent of the amount of tracer 35S-labeled actin added (below the C₅), and the levels of exchange were in agreement with those observed by fluorescence energy transfer (Figs. 5 and 6).

**Exchange of ATP into filaments at steady-state.** An indirect measure of subunit exchange is [32p]ATP incorporation into unlabeled filaments (see Introduction). As...
showed in Fig. 7, the initial rate of $[^{14}]$C$\text{ATP}$ incorporation into filaments was equivalent to ~6% of the F-actin/h, in reasonable agreement with the steady-state ATP hydrolysis rate of 3%/h determined for unlabeled F-actin (Table I). However, the total extent of $[^{14}]$C$\text{ATP}$ incorporation into filaments was limited to ~6% of the F-actin after 2 h of incubation at 25°C. This limited exchange of ATP into filaments is in agreement with the steady-state ATP hydrolysis rate of 3%/h. Because the $C_A$ was constant at 20–30 ng/ml as the total actin concentration decreases below 0.2 mg/ml, the G/F ratio increases dramatically.

**Dependence of Observed Subunit Exchange on F-actin Concentration**

Because the rate and extent of steady-state exchange may depend on filament concentration, $C_A$, and buffering species, the dependence of filament subunit exchange on F-actin concentration under several buffer conditions was studied. The kinetics of subunit exchange as evidenced by fluorescence energy transfer are presented in Fig. 8. At all actin concentrations tested, subunits exchanged with a nearly linear time course until a constant plateau level was reached. The half-time of exchange was ~20 min regardless of actin concentration or type of actin used in the exchange assay. The extent of subunit exchange for both Dictyostelium and muscle actin was quantitated by fluorescence energy transfer and $^{35}$S-labeled actin exchange for actin concentrations ranging from 0.05–2 mg/ml (Fig. 9). All methods used gave similar results over the range of actin concentration examined. At >0.2 mg/ml actin, <10% exchange was observed. Because the $C_A$ was constant at 20–30 ng/ml, as the total actin concentration decreases below 0.2 mg/ml, the G/F ratio increases dramatically.

**FIGURE 6** Subunit exchange between fluorescent-labeled donor and acceptor filaments. IAENS-labeled and FITC-labeled muscle G-actin in TES G-buffer were assembled separately by addition of KCl to 0.1 M, MgCl$_2$ to 1 mM, and ATP to 1 mM. After establishing the baseline fluorescence for 0.2 mg/ml IAENS-labeled F-actin, IAENS-labeled F-actin (0.2 mg/ml final concentration) and FITC-labeled F-actin (0.8 mg/ml final concentration) were mixed and the fluorescence quench was monitored. An initial slight increase in quench was followed by no further changes after 30 min at 25°C. Sonication of the filament mixture produced 56% fluorescence quench, the amount expected for 100% exchange (see Fig. 4). The same result (<5% exchange) was obtained when IAENS-labeled Dictyostelium F-actin was mixed with FM-labeled Dictyostelium F-actin. With FM-labeled actins, maximum quench was 40%.

**FIGURE 7** Incorporation of $[^{14}]$C$\text{ATP}$ into actin filaments. $[^{14}]$C$\text{ATP}$ was gently mixed with muscle F-actin (5 mg/ml) in TES G-buffer containing 0.1 M KCl and 1 mM MgCl$_2$. During incubation at 25°C, 50-$\mu$l aliquots were centrifuged through 20% sucrose. The amount of $[^{14}]$C$\text{ATP}$ in the pelleted actin was measured. The extent of incorporation was 4% after 2 h and 8% after 24 h.

**FIGURE 8** Kinetics of subunit exchange. Rates of exchange between IAENS-labeled donor and FM-labeled acceptor filaments are shown for muscle actin prepared in TES G-buffer containing 20 $\mu$m MgCl$_2$ in place of 50 $\mu$m CaCl$_2$, and assembled in 0.1 M KCl, 1 mM MgCl$_2$, and 1 mM ATP (O); for muscle actin in one part Buffer A diluted into four parts PIPES buffer (final concentrations of $K^+$, Mg$^{2+}$, and ATP were 8.4 mM, 2.0 mM, and 0.44 mM, respectively) (☐); for Dictyostelium actin prepared in TES G-buffer containing 20 $\mu$m MgCl$_2$ in place of 50 $\mu$m CaCl$_2$, and assembled in 0.1 M KCl, 1 mM MgCl$_2$, and 1 mM ATP (●).
Extend of subunit exchange as a function of actin concentration. The extent of subunit exchange was determined by fluorescence energy transfer for muscle actin prepared in TES G-buffer containing 20 μM MgCl₂ in place of 50 μM CaCl₂, and assembled in 0.1 M KCl, 1 mM MgCl₂, and 1 mM ATP (O); for muscle actin in one part Buffer A diluted into four parts PIPES buffer (K⁺, 8.4 mM, Mg²⁺, 2.0 mM, ATP, 0.44 mM final concentrations); for Dictyostelium actin prepared in TES G-buffer containing 20 μM MgCl₂ in place of 50 μM CaCl₂, and assembled in 0.1 M KCl, 1 mM MgCl₂, and 1 mM ATP (A), and into Dictyostelium F-actin in imidazole G-buffer containing 0.1 M KCl, 1 mM MgCl₂, and 1 mM ATP (△). Exchange of [³⁵S]ATP for F-actin bound ADP is also shown (□).

To determine if buffer conditions markedly affect filament subunit exchange, actin was prepared and exchange was measured under several different buffer conditions (Fig. 9). Exchange of actin in buffers containing either TES (pH 7.2), imidazole (pH 7.5), or PIPES (pH 7.0) did not affect actin subunit exchange nor did high (0.1 M) or low (10 mM) levels of K⁺. Furthermore, high free Mg²⁺ (2 mM) or high MgATP (1 mM) did not alter the exchange properties.

We therefore conclude, that under the buffer and salt conditions which were used for these studies, highly purified actin filament subunits undergo only limited exchange at steady state.

DISCUSSION

We have used several experimental techniques to study actin filament subunit exchange in vitro. All have produced highly similar results. Purified actin filaments from either Dictyostelium amoebae or rabbit skeletal muscle when assembled in physiologically related concentrations of K⁺, Mg²⁺, and ATP exhibit the following properties: (a) Actin filaments at >0.5 mg/ml participate in <5% subunit exchange with other filaments or with nonfilamentous actin in equilibrium with filaments. At concentrations of actin below 0.1 mg/ml >20% exchange was observed. Although considerable variability was apparent in this low concentration range due to inherent errors in the methods used, a greater extent of exchange did seem apparent as the total actin approached the C₅. This would be expected since the subunit exchange must go to 100% as the critical concentration is approached. (b) Exchange appears limited to a specific region of the filament since continuous incorporation of subunits up to 100% exchange is not detected. It is likely that limited exchange occurs at filament ends. It cannot be deduced from this study whether limited exchange is an intrinsic property of highly purified individual filaments or derives from the interaction of actin filaments in solution. For example, sites at which filaments cross each other and interact may represent locations at which a vectorial exchange process proceeding from the filament end can terminate. It is also possible that the limited exchange observed here may be due to the presence of two distinct filament populations, one of which cannot undergo exchange while the other exchanges completely. These cases should be most easily analyzed by direct visualization of the exchange of tagged actin monomers into filaments by electron microscopy. (c) Exchange occurs with a half-time of 20–30 min and reaches saturation levels within 60 min at 25°C.

The limited extent of ATP exchange observed here with highly purified actin agrees with the measurements of Gergely, et al. (4). This limited ATP exchange is in quantitative agreement with actin subunit exchange and with ATP hydrolysis. It is clear that ionic conditions can be found that promote complete subunit exchange of purified actin filaments in vitro. Wegner (5) observed nearly 100% subunit exchange in the absence of KCl and in low levels (0.5 mM) of MgCl₂. Wegner and Neuhaus (7) subsequently obtained nearly complete exchange for actin assembled in either 1.2 mM CaCl₂ or 0.6 mM MgCl₂, while only limited exchange occurred in 19 mM KCl (7). Wang and Taylor (27) also found that buffer conditions dramatically affected the rate of actin exchange. In polymerization buffer containing 2.5 mM MgCl₂ and 0.5 mM ATP they detected ~80% subunit exchange with fluorescent labeled actin at 0.2 mg/ml. In contrast, in buffer containing 100 mM KCl, 1 mM MgCl₂, and 1 mM ATP with actin at 0.2 mg/ml Wang and Taylor (27) detected a sixfold decrease in the rate of subunit exchange to ~7% exchange within the first hour. The final extent of exchange in this buffer was not given.

In our experiments in buffers containing 100 mM KCl, 1 mM MgCl₂, and 1 mM ATP, we found no more than 10–20% exchange at an actin concentration of 0.2 mg/ml and <5% at higher actin concentrations. Furthermore, we also observed this same limited exchange for actin polymerized under very similar buffer conditions to those used by Wang and Taylor in their high-exchange case (2.5 mM MgCl₂ and 0.5 mM ATP; see Fig. 9, □). The reason for this difference is not presently known.

The experiments reported here demonstrate that under buffer conditions that approximate ionic concentrations in vivo, exchange of actin filament subunits occurs to only a small and limited extent with the equilibrium nonfilamentous actin pool. It is important to bear in mind that although we conclude from these studies that highly purified actin filaments undergo limited exchange in the presence of physiological concentrations of salts, it is not meant to imply that filaments are incapable of complete exchange in vivo. It seems likely that factor-mediated exchange may be an important biological process. In this regard, fluorescence techniques should serve as powerful tools for studying actin interactions with regulatory proteins. In fact, the fluorescence energy transfer techniques used here have already proven to be a powerful assay for elucidating the sequence of events which follow fragmentation of actin filaments by a 40,000-dalton protein isolated from Dictyostelium (28).
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REFERENCES

1. Asakura, S., and F. Oosawa. 1960. Dephosphorylation of adenosine triphosphate in actin solutions at low concentrations of magnesium. Arch. Biochem. Biophys. 87:273-280.

2. Asakura, S., K. Hotta, N. Imai, T. Ooi, and F. Oosawa. 1957. In: Proceedings of a Conference on the Chemistry of Muscular Contraction from the Committee of Muscle Chemistry of Japan. Igaku Shoin, Ltd. 57.

3. Martonosi, A., M. A. Gouvea, and J. Gergely. 1960. Studies on actin. I. The interaction of 14C-labeled nucleotides with actin. J. Biol. Chem. 235:1700-1703.

4. Gergely, J., M. A. Gouvea, and A. Martonosi. 1960. Studies on actin. II. Partially polymerized actin solutions. J. BioL Chem. 235:1704-1706.

5. Wegner, A. 1976. Head to tail polymerization of actin. Mol. BioL 108:139-150.

6. Huxley, H. 1963. Electron microscopic studies on the structure of natural and synthetic protein filaments from striated muscle. J. BioL Chem. 246:4866-4871.

7. Wegner, A., and J. M. Neuhaus. 1981. Requirement of divalent cation for fast exchange of actin monomers and actin filament subunits. J. Mol. BioL 153:681-693.

8. Conway, E. J. 1957. Nature and significance of concentration relations of potassium and sodium ions in skeletal muscle. Physiol. Rev. 37:S4-S32.

9. Hodgkin, A. L. 1958. Ionic movements and electrical activity in giant nerve fibres. Proc. R. Soc. Lond. B Biol. Sci. 148:3-37.

10. Veesch, R. L., J. W. Randolph Lawson, N. W. Corneli, and H. A. Krebs. 1979. Cytosolic phosphorylation potential. J. Biol. Chem. 254:6538-6547.

11. Scarpa, A., and F. Jack Britton. 1981. Indirect measurements of free cytosolic magnesium ions. Fed. Proc. 40:2684-2692.

12. Simpson, P. A., and J. A. Spudich. 1980. ATP-driven steady-state exchange of monomeric and filamentous actin from Dictyostelium discoideum. Proc. Natl. Acad. Sci. U. S. A. 77:4610-4613.

13. Taylor, D. L., J. Reidler, J. A. Spudich, and L. Stryer. 1981. Detection of actin assembly by fluorescence energy transfer. J. Cell Biol. 89:362-367.

14. Stryer, L. 1978. Fluorescence energy transfer as a spectroscopic ruler. Annu. Rev. Biochem. 47:819-846.

15. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. J. Biol. Chem. 246:4566-4571.

16. Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. In Methods in Enzymology 65, Part B. D. L. Frederiksen and L. W. Cunningham, editors. Academic Press, Inc., New York.

17. MacLean-Fletcher, S., and T. D. Pollard. 1980. Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. Biochim. Biophys. Res. Commun. 3:18-27.

18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.

19. Uyemura, D. G., S. S. Brown, and J. A. Spudich. 1978. Biochemical and structural characterization of actin from Dictyostelium discoideum. J. Biol. Chem. 253:9008-9009.

20. Tso, T., and J. Cho. 1979. Fluorescence lifetime quenching studies on the accessibilities of actin sulfhydryl sites. Biochemistry. 18:2759-2763.

21. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

22. Sugano, J., and Y. Miyoshi. 1964. The specific precipitation of orthophosphate and some other chemical applications. J. Biol. Chem. 239:2380-2384.

23. Clark, M., and J. A. Spudich. 1974. Biochemical and structural studies of actomyosin-like proteins from non-muscle cells. J. Mol. Biol. 66:209-222.

24. Honk, T. W., Jr., and K. Ue. 1974. The measurement of actin concentration in solution: a comparison of methods. Anal. Biochem. 62:66-71.

25. Porter, M., and A. Weber. 1979. Non-cooperative response of actin-cysteine 373 in cooperatively behaving regulated actin filaments. FEBS (Fed. Eur. Biochem. Soc.) Lett. 105:259-262.

26. Frieden, C., D. Lieberman, and H. R. Gilbert. 1980. A fluorescent probe for conformational changes in skeletal muscle G-actin. J. Biol. Chem. 255:8991-8993.

27. Wang, Y., and D. L. Taylor. 1981. Probing the dynamic equilibrium of actin polymerization by fluorescence energy transfer. Cell. 27:429-436.

28. Yamamoto, K., J. D. Pardee, J. Reidler, L. Stryer, and J. A. Spudich. 1982. The mechanism of interaction of Dictyostelium sevires with actin filaments. J. Cell Biol. In press.