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 ^{35}S-labeled actin monomers into unlabeled actin filaments, and exchange of [^{14}C]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 ^{35}S-labeled *Dictyostelium* actin monomers into unlabeled muscle actin or *Dictyostelium* actin filaments. Furthermore, the proportion of filament-bound ADP which exchanged with [^{14}C]-ATP was nearly the same as actin subunit exchange measured by fluorescence energy transfer and ^{35}S-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, and co-workers (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 hyrolysis 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 [^{14}C]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 ^{14}C-nucleotide per mol of F-actin subunits. However, in [^{14}C]ATP experiments by Gergely et al. (4) an average of only 11% nucleotide exchange was observed in filaments polymerized in 0.7 mM $MgCl_2$ 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 $MgCl_2$ (0.5 mM), or in 1.2 mM $CaCl_2$.

Since intracellular concentrations of K^+ are generally believed to be in the 100 mM range (8, 9), and Mg^{2+} and ATP concentrations of 1–5 mM are expected (10, 11), we wished to

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 AUGUST 1982 316-324

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, ^{35}S-labeled actin monomer exchange into filaments (12) and fluorescence energy transfer between neighboring actin subunits within a filament (13, 14). Furthermore, [^{14}C]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[1], pH 8.0 at 25°C, 0.2 mM Na_2ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 0.005% NaN_3.

IAENS, FM labeling buffer: 10 mM K_2HPO_4, pH 8.0 at 4°C, 0.2 mM Na_2ATP, 0.005% NaN_3, 0.1 M KCl, 1 mM $MgCl_2$.

FITC labeling buffer: 50 mM K_2CO_3, 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, 50μM $CaCl_2$, 0.005% NaN_3 (contains ~2 mM K^+ added as KOH to titrate 2 mM TES to pH 7.2. **NOTE:** In some experiments 20 μM $MgCl_2$ was substituted for 50 μM $CaCl_2$. 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% NaN_3.

PIPES buffer: 10 mM PIPES, pH 7.0 at 25°C, 0.5 mM ATP, 0.5 mM dithiothreitol, 2.5 mM $MgCl_2$ (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*, 1.5 h, at 4°C, and repolymerization of the clarified supernatant G-actin with 0.1 M KCl, 1 mM $MgCl_2$, 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 $MgCl_2$ 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 discoideum 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* at 4°C for 1.5 h, and reassembled by addition of 0.1 M KCl and 1 mM $MgCl_2$. *Dictyostelium* ^{35}S-labeled actin, radiolabeled in vivo by the procedure of Simpson and Spudich (12), was isolated by the method of Uyemura, et al. (19), and was further purified by the recycle method used for unlabeled *Dictyostelium* actin.

Labeling of F-actin with IAENS or FM

Typically, 12 mg of freshly prepared DEAE-treated, gel-filtered muscle F-actin was incubated for 3–4 h with 5 mM 2-mercaptoethanol, centrifuged at 150,000 *g* for 1.5 h at 4°C, and the pelleted actin homogenized into degassed IAENS, FM labeling buffer. Homogenates were dialyzed overnight in 10,000-dalton cutoff collodion bags (Schleicher and Schuell, Keene, NH) at 4°C against 1 liter of the same buffer, and the dialyzed actin diluted to 2 mg/ml with buffer. A stock solution of 120 mM IAENS (Molecular Probes, Plano, TX) was prepared by dissolving 11 mg in 200 μl of dimethylformamide. ~20 μl of 2 N KOH was added to bring the final pH to 8. FM (Molecular Probes, Plano, TX) was prepared by dissolving 11 mg in 200 μl of dimethylformamide plus 15 μl of 2 N KOH. F-actin and fluorescent label stock solutions were kept chilled on ice, and a 20:1 molar excess of label was mixed into the F-actin solution. The mixture (final pH, 8) was incubated for 60–70 h on ice in the dark. Labeled F-actin was sedimented at 150,000 *g* at 4°C for 1.5 h, gently homogenized into TES G-buffer containing 0.1 M KCl and 1 mM $MgCl_2$, dialyzed overnight in collodion bags in the dark at 4°C to remove residual unbound label, and stored on ice in the dark. Labeling stoichiometries of 0.8–1.1 mols FM or IAENS per mol actin were routinely achieved with ~40–50% recovery of actin from the labeling procedure. IAENS is known to label cys 373 specifically (20).

Labeling of F-actin with FITC

Purified muscle F-actin at 2 mg/ml (10–15 ml) was dialyzed in 10,000-dalton cutoff collodion bags against 1 liter of FITC labeling buffer for 4 h at 4°C. FITC (isomer I; Molecular Probes, Plano, TX) stock solution (160 mM) was prepared by the addition of 77 mg to 1 ml ethanol plus 0.24 ml of 2 N KOH to obtain a pH of ~9. The FITC stock solution was added in a 200:1 molar ratio to F-actin on ice. A final pH of 9.3–9.4 was obtained by titration with 0.1 N KOH or HCl. The mixture was incubated for 24 h on ice in the dark. The labeled F-actin was sedimented at 150,000 *g* for 1.5 h at 4°C, gently homogenized into TES G-buffer containing 0.1 M KCl and 1 mM $MgCl_2$, dialyzed 24 h at 4°C against 1 liter of buffer to remove unbound FITC, and stored on ice. ~50% recovery of labeled actin was obtained which contained 0.8–1.0 mol FITC per mol actin. Lower FITC/actin labeling stoichiometries (0.3–0.7) resulted when the labeling mixture contained lower molar ratios of FITC to unlabeled actin (50:1 to 150:1).

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 $MgCl_2$). 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 I, and no increase in critical concentration nor decrease in viscosity occurred in the time course of the exchange experiments.

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 $MgCl_2$ 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 actin samples (100 μl) were sedimented for 1 h at 150,000 *g* in an airfuge (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 (C_A) 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 I.

ASSEMBLY KINETICS: Assembly of recycled fluorescent-labeled muscle

TABLE I

Assembly Properties of Fluorescent-Labeled Actin

F-actin	C_A	$t_{1/2}$	Mol ATP hydrolyzed per mol actin during assembly	Mol ATP hydrolyzed per mol actin per h at steady-state
	$\mu g/ml$*	*min*		
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 $MgCl_2$. C_A determinations at 1 mg/ml gave nearly identical values.

[1] Abbreviations used: TRIS: tris(hydroxymethyl)aminomethane; IAENS: 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid; FM: fluorescein-5-maleimide; FITC: fluorescein-5-isothiocyanate (isomer I); TES: (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid); PIPES: (piperazine-N,N′-bis[2-ethane sulfonic acid]); SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; DEAE: diethylaminoethyl cellulose; C_A: critical actin concentration.

actin or unlabeled muscle actin at 1 mg/ml in TES G-buffer was initiated by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM. Assembly was followed in a Cannon-Manning 150 viscometer (Cannon Instrument Co., State College, PA) having a buffer outflow time of 30 s. Coassembly of 0.8 mg/ml FITC-labeled actin with 0.2 mg/ml IAENS-labeled actin was followed by the decrease in IAENS fluorescence at 470 nm (13). Comparative assembly kinetics are presented in Table I.

HYDROLYSIS OF ATP DURING ASSEMBLY AND AT STEADY-STATE: Levels of ATP hydrolysis during assembly and at steady-state equilibrium of F-actin were compared for unlabeled muscle actin, IAENS-labeled actin, FITC-labeled actin, and a 4:1 mixture of FITC-:IAENS-labeled actin (Table I). Recycled G-actins (1 ml, 1 mg/ml) in TES G-buffer were incubated for 3 h on ice with 15 μl of [γ-^{32}P]-ATP (Amersham, Arlington Heights, IL) to obtain an activity of ~20,000 cpm/μl of G-actin solution. Samples were brought to 25°C constant temperature and assembly initiated by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM. Duplicate 10-μl aliquots were removed from the assembly mix at 2-min intervals during assembly, or at 30-min intervals for up to 5 h at steady-state equilibrium, and assayed for the hydrolysis of [γ-^{32}P]-ATP by the modified method of Sugino and Miyoshi (22) as described by Clarke and Spudich (23). Nucleotide and actin concentrations were determined spectroscopically using the extinction coefficients $\epsilon^{M}_{259} = 15{,}400$ for nucleotide and $\epsilon^{0.1\%}_{290} = 0.62$ (24) for actin.

Fluorescence Characterization of Labeled Actin

LABELING STOICHIOMETRY: The concentration and dye-to-protein ratios of fluorescent-labeled actins were determined spectrophotometrically in a Beckman 35 scanning spectrophotometer (Beckman Instruments, Menlo Park, CA). The extinction coefficients of fluorescent labels were determined in TES G-buffer at pH 7.2 and in 10 M urea at pH 12. In TES G-buffer, extinction coefficients were: IAENS, $\epsilon^{M}_{335} = 5.1 \times 10^3\ cm^{-1}$; FITC, $\epsilon^{M}_{490} = 6.4 \times 10^4\ cm^{-1}$; and FM, $\epsilon^{M}_{490} = 6.0 \times 10^4\ cm^{-1}$. In 10 M urea at pH 12 extinction coefficients were: IAENS, $\epsilon^{M}_{340} = 5.7 \times 10^3\ cm^{-1}$; FITC, $\epsilon^{M}_{498} = 6.6 \times 10^4\ cm^{-1}$; and FM, $\epsilon^{M}_{498} = 7.6 \times 10^4\ cm^{-1}$. To determine dye to actin labeling stoichiometries, actin samples of known concentration in TES G-buffer and in TES G-buffer containing 10 M urea at pH 12, were scanned from 600 to 250 nm, and the concentration of bound dye was calculated from the extinction coefficients in each solvent. Labeling stoichiometries were 0.8–1.0 for all labeled actins regardless of the solvent condition used for the determination. Fluorescence measurements of samples in 3 x 3 mm quartz cuvettes (Precision Cell, Hicksville, NY) were carried out using a Spex Fluorolog fluorometer (Spex Industries, Inc., Metuchen, NJ) with excitation and emission bandpass set at 10 nm.

ENERGY TRANSFER EFFICIENCY: A fluorescence energy transfer quench curve was obtained as a function of the mole fraction of acceptor-labeled (FITC) actin in filaments coassembled with donor- and acceptor-labeled G-actins. IAENS-labeled actin in TES G-buffer was added to a mixture containing various proportions of FITC-labeled actin and unlabeled G-actin, and assembly was initiated by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM. Final concentrations were 0.2 mg/ml IAENS-labeled actin and 0.8 mg/ml of combined FITC-labeled and unlabeled actins. Assembly was followed by the decrease in IAENS fluorescence at 470 nm (excitation wavelength, 340 nm). Percent fluorescence quench was calculated after assembly was complete.

$$\text{Percent quench} = \frac{F_G - F_F}{F_G} \times 100$$

where F_F = Fluorescence of coassembled F-actin mixture at 470 nm and F_G = Fluorescence of G-actin mixture at 470 nm.

Quench curves were also obtained from the increase in fluorescence which followed sonication of coassembled IAENS-, FITC-labeled filaments with increasing concentrations of unlabeled F-actin. Filaments coassembled with four parts FITC-labeled actin to one part IAENS-labeled actin were mixed with unlabeled F-actin to achieve final concentrations of 0.2 mg/ml fluorescent-labeled actin and from 0 to 2.2 mg/ml unlabeled actin. Sonication causes complete randomization of fluorescent filament subunits and unlabeled filament subunits. Consequently, donor-labeled subunits are separated from acceptor-labeled subunits by intervening unlabeled subunits, and a decrease in quench (increase in donor fluorescence) occurs. Percent quench due to the randomization of filament subunits by sonication was determined by fluorescence at 470 nm from the relationship:

$$\text{Percent quench} = \frac{F_G - F_{sonicate}}{F_G - F_F} \times 100$$

where F_G = fluorescence of the IAENS- plus FITC-labeled G-actin mixture, F_F = fluorescence of the IAENS-, FITC-labeled coassembled filaments mixed with unlabeled filaments before sonication, and $F_{sonicate}$ = fluorescence of the IAENS-, FITC-labeled coassembled filaments mixed with unlabeled filaments after sonication. The IAENS-, FITC-labeled G-actin fluorescence was corrected for dilution by assembly salts and by unlabeled filaments, and a quench curve obtained.

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 1.0 mg/ml in 0.3 ml. 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, $MgCl_2$ stock solution to achieve final concentrations of 0.1 M KCl and 1 mM $MgCl_2$. 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 coassembled F-actin sample was withdrawn from the cuvette, sonicated on ice for 30 s to 1 min with a Kontes micro-ultrasonic cell disrupter (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 quantitate 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-, 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:

$$\text{Percent subunit exchange} = \frac{F_{final} - F_{initial}}{F_{sonicate} - F_{initial}} \times 100$$

where $F_{initial}$ = fluorescence of the filament mixture immediately after mixing, F_{final} = fluorescence of the filament mixture after the exchange period, and $F_{sonicate}$ = 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 measurement 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 $MgCl_2$ 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 decreases 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 redetermined 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 ($F_{initial}$), at the end of the exchange period (F_{final}), and after sonication ($F_{sonicate}$).

$$\text{Percent subunit exchange} = \frac{F_{initial} - F_{final}}{F_{initial} - F_{sonicate}} \times 100$$

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^+, Mg^{2+}, and ATP concentrations. Specific buffer conditions, actin concentrations, and actin sources are given in the figure legends.

INCORPORATION OF ^{35}S-LABELED ACTIN MONOMER INTO FILAMENTS: ^{35}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 μl of 0.25 mg/ml ^{35}S-labeled G-actin (sp act = 3,000 cpm/μg) 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_2O, dispersed in 10 ml of ACS scintillant (Amersham, Arlington Heights, IL), and counted. Percent exchange of F-actin subunits with ^{35}S-labeled actin monomer was calculated from the distribution of ^{35}S-labeled actin specific activity between sedimentable (F) and nonsedimentable actin (C_A):

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

$$= \frac{\text{Sp. act. of F-actin}}{\text{Sp. act. of } C_A} \times 100$$

The concentration of actin remaining in the 150,000 *g* supernatant was determined on three 20-μl aliquots by Bradford assay (21).

To determine the assembly competence of ^{35}S-labeled actin remaining in the supernatant fraction after complete exchange, 100 μl of supernatant fraction was mixed with 200 μl 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. The resulting supernatant was decanted and counted. The cpm remaining in this assembly supernatant reflect the amount of ^{35}S-labeled actin that is not competent to assemble (or presumably to exchange). Consequently assembly-incompetent counts were subtracted from the "cpm in supernatant" value in the percent subunit exchange calculation. Typically this value was ~10% of the C_A.

INCORPORATION OF [^{14}C]ATP INTO FILAMENTS AT STEADY-STATE: Rabbit muscle F-actin was sedimented at 150,000 *g* for 1.5 h at 4°C, and the pelleted actin 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 4°C, and repolymerized by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM. [^{14}C]ATP (sp act = 524 mCi/mmol) (Amersham, Arlington Heights, IL) was added to the F-actin to give 1,500 cpm/μl of sample. Samples were mixed by five passes 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 [^{14}C]ATP at various times after mixing, 50-μl aliquots of the incubation mix were gently layered over 50 μl 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 [^{14}C]ATP 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 μl of H_2O and counted. Control experiments in which 5 μl of [^{14}C]ATP were placed onto the F-actin which had been overlayered on the sucrose cushion demonstrated that 99% of the free [^{14}C]ATP 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:

$$\text{Percent of F-actin subunits incorporating ATP} = \frac{(\text{cpm}_{\text{pellet}}/\text{cpm}_{\text{total}})[\text{ATP}_{\text{free}}]}{[\text{Actin}_{\text{total}}]} \times 100$$

The critical actin concentration in this experiment was 20 μg/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

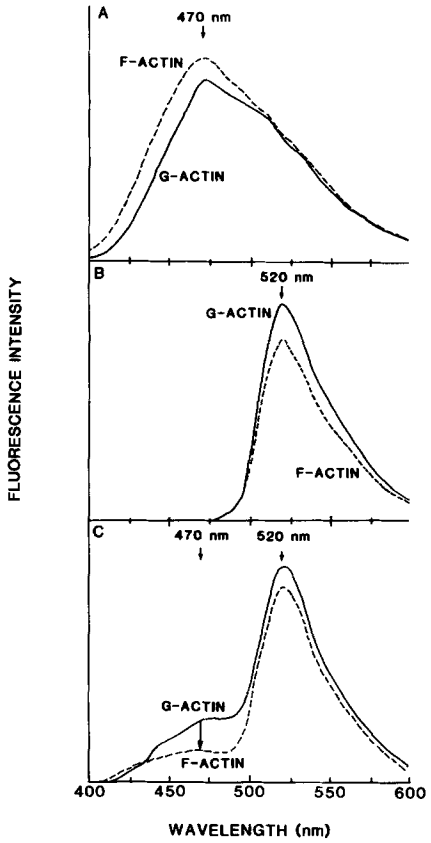

FIGURE 1 Fluorescence emission spectra of IAENS- and FITC-labeled muscle actin. (*A*) IAENS-labeled muscle G-actin (——) at 0.1 mg/ml in TES G-buffer with a dye to protein ratio of 1.0 exhibited maximum fluorescence at 470 nm. IAENS-labeled muscle F-actin (- - -), prepared by the addition of 0.1 M KCl and 1 mM $MgCl_2$, exhibited an 11% increase in fluorescence intensity at 470 nm. (*B*) FITC-labeled muscle G-actin (——) at 0.2 mg/ml in TES G-buffer with a dye to protein ratio of 0.9 exhibited maximum fluorescence at 520 nm. FITC-labeled muscle F-actin (- - -), prepared by the addition of 0.1 M KCl and 1 mM $MgCl_2$, exhibited a 15–20% decrease in fluorescence intensity at 520 nm. (*C*) A mixture of four parts FITC-G-actin with one part IAENS-G-actin (——) (total actin concentration, 0.2 mg/ml) exhibited fluorescence maxima at 475 nm and 520 nm. All of the emission at 470 nm comes from IAENS-labeled actin. Coassembly 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.

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 occurs (Fig. 1 *A*) in agreement with the observations of Porter and Weber (25) and Frieden et al. (26). The FITC-labeled actin spectrum exhibits a decrease in fluorescence intensity of ~17% at 520 nm (Fig. 1 *B*). Assembly of a mixture of IAENS- plus FITC-labeled actins is accompanied by a very large decrease of IAENS fluorescence at 470 nm (Fig. 1 *C*). This fluorescence quenching is due to energy transfer

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 ≤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 ~55% quench at all F-actin concentrations used when the mole fraction of FITC-labeled actin is 0.60–0.65.

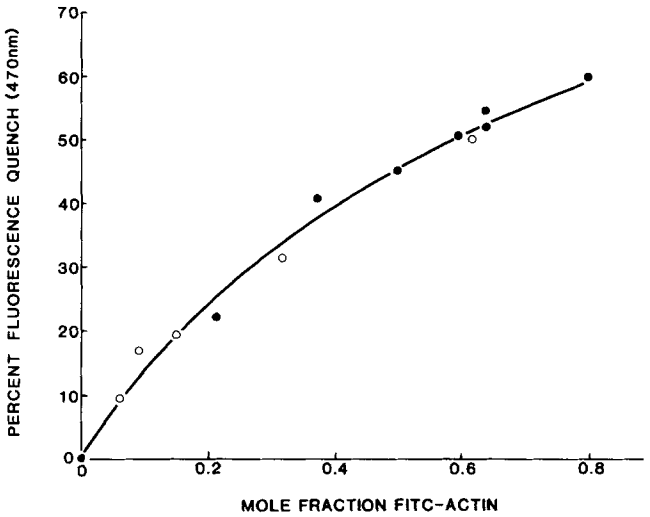

FIGURE 2 Efficiency of energy transfer between IAENS-labeled actin and FITC-labeled actin. Mixtures of IAENS-labeled, FITC-labeled, and unlabeled muscle G-actin were coassembled by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM (○). Samples consisted of a constant amount of IAENS-labeled actin (0.2 mg/ml) and 0.8 mg/ml of mixtures of FITC-labeled + unlabeled actin containing an increasing proportion of FITC-labeled actin. Percent quench was calculated from the final fluorescence obtained after coassembly (See Materials and Methods). Percent quench resulting from sonication of previously coassembled IAENS-, FITC-labeled filaments with increasing concentrations of unlabeled F-actin is also shown (●). Identical quench curves are obtained when fluorescent-labeled subunits are randomized within filaments by either coassembly or sonication.

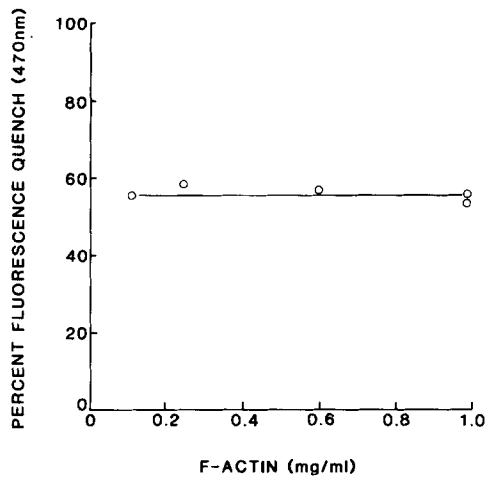

FIGURE 3 Effect of filament concentration on fluorescence quench. Muscle FITC-labeled G-actin and IAENS-labeled G-actin in TES G-buffer were mixed to obtain final total actin concentrations of 0.1 to 1 mg/ml. The mole fraction of acceptor (FITC-labeled actin) was 0.60–0.65 in all samples. Mixtures were assembled at 25°C by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM. Critical actin concentrations (C_A) 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 C_A.

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 2.0 mg/ml for assembly with 0.1 M KCl + 1 mM $MgCl_2$. 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-

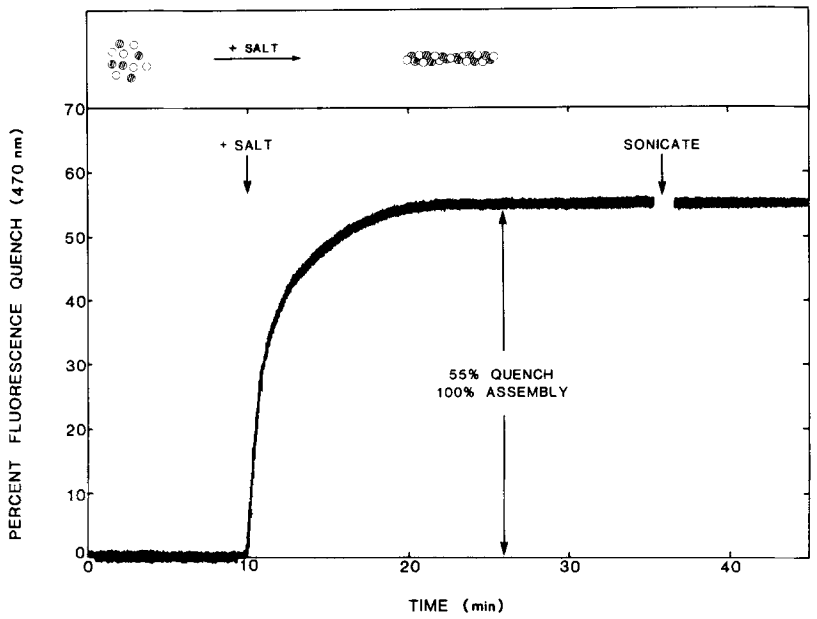

FIGURE 4 Detection of actin assembly by fluorescence energy transfer. Rabbit skeletal muscle IAENS-labeled G-actin (0.2 mg/ml final concentration) in TES G-buffer was mixed with FITC-labeled G-actin (0.8 mg/ml final concentration, mole fraction FITC-labeled actin, 0.65). The fluorescence emission was 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, $MgCl_2$ 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.

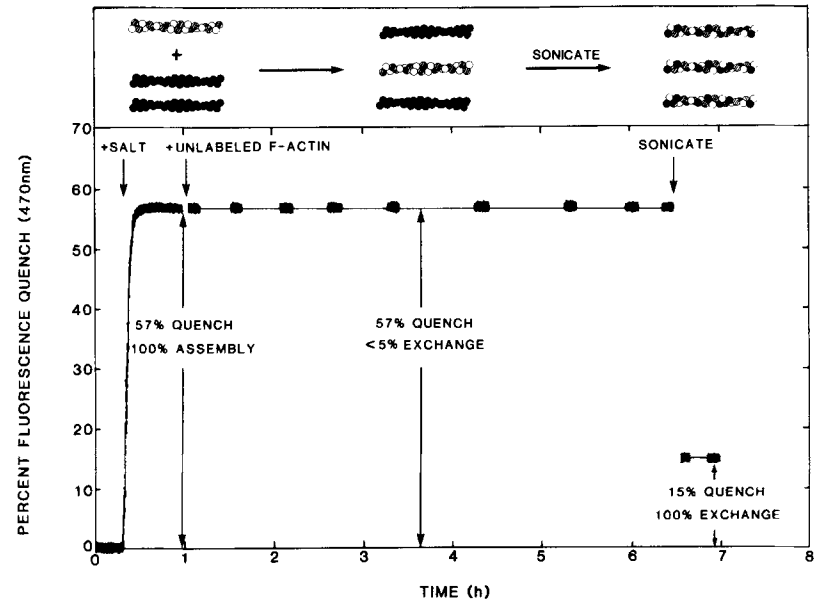

FIGURE 5 Subunit exchange between fluorescent-labeled filaments and unlabeled filaments. Muscle IAENS-labeled G-actin (0.2 mg/ml) and FITC-labeled G-actin (1.2 mg/ml) in TES G-buffer were coassembled with 0.1 M KCl + 1 mM $MgCl_2$ + 1 mM ATP. Coassembled filaments containing a mole fraction FITC-labeled 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 *Dictyostelium* F-actin. In this case, 35% maximum quench was observed for coassembled IAENS, FM filaments.

crease 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. 6). 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 as the acceptor actin. In this case a maximum quench of 40% was obtained for fully coassembled actin or sonicated mixtures of IAENS-labeled F-actin plus FM-labeled F-actin at an FM-labeled actin mole fraction of 0.6. Mixtures of IAENS-labeled filaments and FM-labeled filaments also demonstrated <5% subunit exchange at an actin concentration of 1 mg/ml.

EXCHANGE OF ^{35}S-LABELED G-ACTIN INTO UNLABELED FILAMENTS: A complementary method of measuring the extent of filament subunit exchange between actin monomers and filaments at steady state involves incorporation of ^{35}S-labeled actin monomers from *Dictyostelium* 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), ^{35}S-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 ^{35}S-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 ^{35}S-labeled actin after the 3-h incubation in the exchange assays (see Materials and Methods) demonstrated that >90% of the unexchanged ^{35}S-labeled actin was still capable of assembly. Percent exchange was independent of the amount of tracer ^{35}S-labeled actin added (below the C_A), 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 [^{14}C]ATP incorporation into unlabeled filaments (see Introduction). As

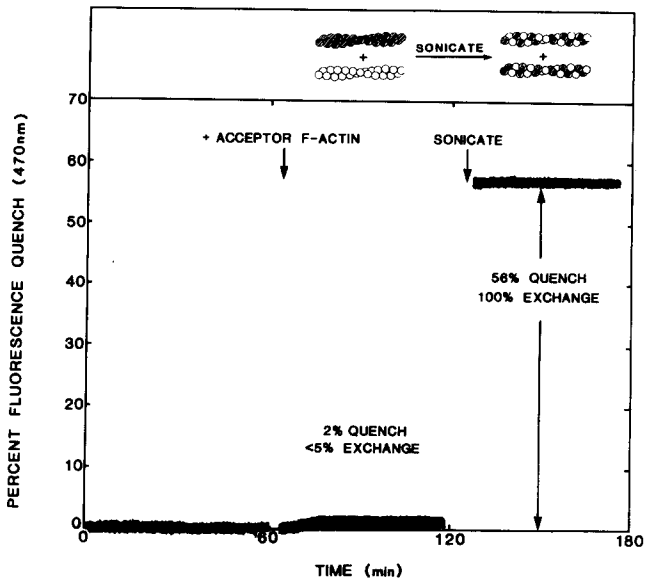

FIGURE 6 Subunit exchange between fluorescent-labeled donor and acceptor filaments. IAENS-labeled and FITC-labeled muscle G-actin in TES G-buffer were assembled separately by addition of KCl to 0.1 M, $MgCl_2$ to 1 mM, and ATP to 1 mM. After establishing the baseline fluorescence for 0.2 mg/ml IAENS-labeled 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%.

shown in Fig. 7, the initial rate of [^{14}C]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]ATP incorporation into filaments was limited to 4% of the F-actin after 2 h of incubation and did not exceed 8% of the F-actin after 24 h of incubation at 25°C. This limited exchange of ATP into filaments is in agreement with the amount of subunit exchange determined by fluorescence energy transfer and by ^{35}S-labeled actin incorporation into filaments at steady-state.

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 μg/ml, as the total actin concentration decreases below 0.2 mg/ml, the G/F ratio increases dramatically.

TABLE II

Exchange of ^{35}S-labeled Actin into Filaments

^{35}S-actin monomer	Unlabeled actin C_A*	Unlabeled F-actin	Cpm in supernatant‡	Cpm in pellet	Exchange§
μg/ml	μg/ml	μg/ml			%
0.5	12	990	220	340	1.9
1.0	17	980	410	560	2.4
10.0	19	980	5300	4800	1.8

* The critical concentration was determined by sedimentation (see Materials and Methods).

‡ Corrected for nonpolymerizable ^{35}S-labeled actin (<10%; see Materials and Methods).

§ Percent (%) exchange was calculated as described in Materials and Methods.

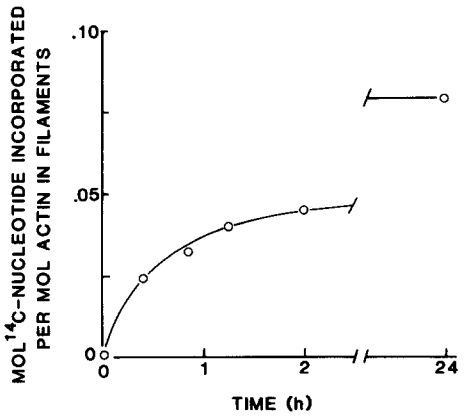

FIGURE 7 Incorporation of [^{14}C]ATP into actin filaments. [^{14}C]ATP was gently mixed with muscle F-actin (5 mg/ml) in TES G-buffer containing 0.1 M KCl and 1 mM $MgCl_2$. During incubation at 25°C, 50-μl aliquots were centrifuged through 20% sucrose. The amount of [^{14}C]ATP in the pelleted actin was measured. The extent of incorporation was 4% after 2 h and 8% after 24 h.

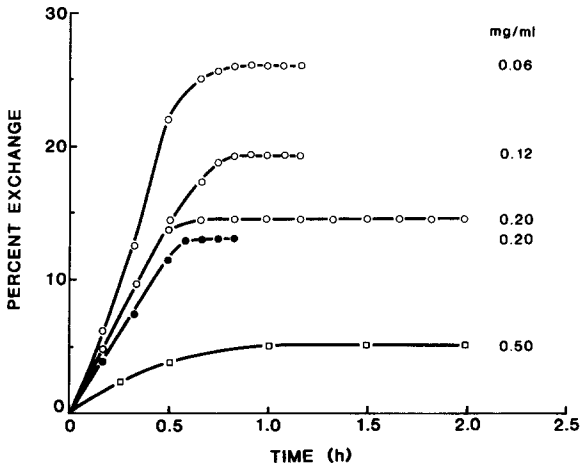

FIGURE 8 Kinetics of subunit exchange. Rates of exchange between IAENS-labeled donor and FM-labeled acceptor filaments are shown for muscle actin prepared in TES G-buffer containing 20 μM $MgCl_2$ in place of 50 μM $CaCl_2$, and assembled in 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (○); for muscle actin 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 μM $MgCl_2$ in place of 50 μM $CaCl_2$, and assembled in 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (●).

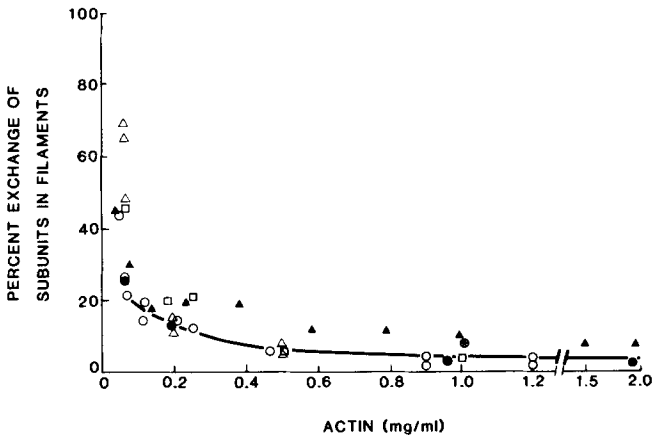

FIGURE 9 Extent of subunit exchange as a function of actin concentration. The extent of subunit exchange was determined by fluorescence energy transfer for muscle actin prepared in TES G-buffer containing 20 μM $MgCl_2$ in place of 50 μM $CaCl_2$, and assembled in 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (○); for muscle actin in one part Buffer A diluted into four parts PIPES buffer (K^+, 8.4 mM, Mg^{2+}, 2.0 mM, ATP, 0.44 mM final concentrations) (□); for *Dictyostelium* actin prepared in TES G-buffer containing 20 μM $MgCl_2$ in place of 50 μM $CaCl_2$, and assembled in 0.1 M KCl, 1 mM $MgCl_2$ (●). In these experiments exchange was measured by mixing donor-labeled (IAENS) filaments with acceptor-labeled (FM) filaments (see Materials and Methods). Subunit exchange was also determined by incorporation of ^{35}S-labeled *Dictyostelium* G-actin into unlabeled muscle F-actin in imidazole G-buffer containing 0.1 M KCl, 1 mM $MgCl_2$ and 1 mM ATP (△), and into *Dictyostelium* F-actin in imidazole G-buffer containing 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (▲). Exchange of [^{14}C]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 Mg^{2+} (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^+, Mg^{2+}, 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 C_A. 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 $MgCl_2$. Wegner and Neuhaus (7) subsequently obtained nearly complete exchange for actin assembled in either 1.2 mM $CaCl_2$ or 0.6 mM $MgCl_2$, 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 $MgCl_2$ 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 $MgCl_2$, 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 $MgCl_2$, 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 $MgCl_2$ 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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