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Dynamic exchange of myosin molecules between thick filaments

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ABSTRACT **To examine thick filament assembly and myosin exchange, a fluorescence energy transfer assay has been established. Assembly-competent myosin molecules labeled with the sulfhydryl-specific fluorochromes 5-{2-[(iodoacetyl)-amino]ethyl}aminonaphthalene-1-sulfonic acid (IAEDANS) or 5-iodoacetamidofluorescein (IAF) were prepared. Using IAEDANS-labeled myosin as fluorescence donor and IAF-labeled myosin as acceptor, thick filament formation was followed by the decrease in donor fluorescence at 0.1 M KCl/10 mM potassium phosphate, pH 6.9. The critical concentration of myosin—i.e., that concentration that remained unassembled at equilibrium with fully formed filaments—was 40 nM. In FET and ^{125}I-labeled myosin incorporation assays, extensive exchange of myosin between thick filaments was observed. The presence of a critical concentration and the measurements of extensive exchange suggest a dynamic equilibrium between fully polymerized myosin and a small pool of soluble myosin.**

Skeletal muscle thick filaments have been shown to be polymers of myosin (1) associated with several accessory proteins, such as C-protein (2–4), M-protein (5), myomesin (6), and end protein (7). Myosin tails make up the backbone of the filaments, while the myosin heads crossbridge myosin filaments to actin-containing thin filaments (1, 8). Myosin molecules have the ability to interact with each other in both parallel and antiparallel arrays. The central portion of the thick filament (the bare zone) contains myosin molecules in an antiparallel array; the remainder of the thick filament is composed of myosin molecules in parallel alignment. The self-assembly properties of myosin have been examined with a variety of *in vitro* assays [reviewed by Harrington and Rodgers (9)], which include high-speed and differential velocity sedimentation (10–13), pressure jump (14–16), light scattering (17), and electric birefringence (18). These studies have demonstrated that thick filaments dissolve in high ionic-strength solution; if the ionic strength is then lowered, the myosin monomers spontaneously reassemble into bipolar filaments that resemble, but are not identical to, the native structure. The mechanism of the assembly events and their relationship to *in vivo* events are still uncertain. Early embryonic forms of myosin must assemble in the absence of preexisting myofibrils; later in development and in the adult, other isoforms are added to the sarcomere by replacement of either individual molecules or removal of entire thick filaments. It is probable that thick filament turnover occurs by a process that does not disrupt myofibrillar function or sarcomere organization, but little is known about these turnover reactions (19). Since microinjected, fluorescently labeled actin (20–23), tropomyosin (20, 21), and α-actinin (20, 21) are rapidly incorporated into the myofibrils of cultured muscles, it is likely that monomeric proteins are in an exchange equilibrium with polymeric proteins in the filaments. It is not known if myosin molecules participate in such an exchange reaction or whether a monomeric pool of this protein exists in embryonic or adult muscle.

In this study we have examined thick filament assembly and myosin exchange using a fluorescence energy transfer (FET) assay. The FET assay is based on the observation (24) that a donor fluorochrome, when excited at the proper wavelength, transfers resonance energy to an acceptor fluorochrome. The efficiency of transfer is proportional to the inverse 6th power of the distance between acceptor and donor. In practice, this distance is approximately 7 nm or less. Successful FET requires fluorochromes with large quantum yields and donor–acceptor pairs that have significant spectral overlap (24). If these criteria are met, FET becomes a highly sensitive technique for examining protein–protein interactions. With contractile systems, FET has been used successfully to examine actin polymerization and to determine the mechanism of subunit exchange between actin filaments in the absence (25–28) and presence (29) of a filament-severing protein. We have prepared assembly-competent myosin molecules labeled with the sulfhydryl-specific fluorochrome 5-{2-[(iodoacetyl)amino]ethyl}aminonaphthalene-1-sulfonic acid (IAEDANS) or 5-iodoacetamidofluorescein (IAF) and followed thick filament formation by the decrease in donor fluorescence. The critical concentration of myosin—i.e., that concentration that remains unassembled at equilibrium with fully formed filaments—was 40 nM. Extensive exchange of myosin between thick filaments was observed, suggesting a dynamic equilibrium between polymerized myosin and a small but kinetically active pool of monomers or soluble oligomers. Preliminary reports of this work have appeared (30, 31). Evidence for exchange of myosin between filaments of smooth muscle has been reported in abstract form (32).

MATERIALS AND METHODS

Myosin Purification and Labeling with Fluorochromes. Myosin was isolated from the pectoralis muscle of adult White Leghorn chickens by the method of Bader *et al.* (33). The purified myosin was precipitated by dialysis for 16 hr against low-salt buffer (0.05 M KCl/10 mM potassium phosphate, pH 6.5) at 4°C sedimented at 10,000 × *g* for 20 min, and resuspended in a high-salt buffer (0.5 M KCl/10 mM potassium phosphate, pH 7) at 4°C. The resulting myosin solution was clarified at 10,000 × *g* for 20 min and either used immediately or stored in high-salt buffer containing 50% (vol/vol) glycerol and 1 mM dithiothreitol at −20°C.

The fluorescent labeling of myosin was a modification of several procedures previously used for the labeling of F-actin (25, 28) and myosin (34, 35). The fluorochromes used were the sulfhydryl-specific probes IAEDANS and IAF (Molecular Probes, Junction City, OR). Stock solutions of 100 mM were prepared by dissolving fluorochromes in dimethylformamide with 1 M KOH added to bring the final pH to 7.

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Abbreviations: FET, fluorescence energy transfer; IAEDANS, 5-{2-[(iodoacetyl)amino]ethyl}aminonaphthalene-1-sulfonic acid; IAF, 5-iodoacetamidofluorescein; HMM and LMM, heavy and light meromyosins.

Myosin filaments were formed by dialyzing myosin (5 mg/ml) against filament buffer (0.1 M KCl/10 mM potassium phosphate, pH 6.9) with fluorochrome added at a 50:1 molar excess of label. This mixture was incubated for 24 (IAF) or 72 (IAEDANS) hr on ice in the dark. The reaction was terminated with 1 mM dithiothreitol, and free label was removed by dialysis against a high-salt buffer at pH 7.4, which also causes disassembly of the filaments. Fluorochrome-labeled myosin was then recycled by dialysis against the low-salt buffer at 4°C, sedimentation, and resuspension in high salt buffer.

Characterization of Fluorochrome-Labeled Myosin. The dye-to-protein ratios of labeled myosin were determined spectrophotometrically in a Perkin–Elmer λ-3 scanning spectrophotometer. The extinction coefficients of fluorescent labels were determined in 10 M urea to be $\epsilon_{335} = 5.3 \times 10^3$ $M^{-1} \cdot cm^{-1}$ for IAEDANS and $\epsilon_{490} = 7.6 \times 10^4$ $M^{-1} \cdot cm^{-1}$ for IAF. To establish dye-to-myosin ratios, known concentrations of labeled myosin diluted in 10 M urea were scanned from 600 to 250 nm, and the concentration of bound fluorochrome was calculated from the extinction coefficients.

The intramolecular distribution of fluorescent label was established by chymotryptic digestion of labeled myosin (10 mg/ml) carried out for 10 min by the method of Weeds and Pope (36). Digestion under low-salt conditions with myosin filaments resulted in the production of Rod (tail-containing) and S1 (head-containing) fragments, whereas the same procedure carried out under high-salt conditions with soluble myosin yielded heavy meromyosin (HMM) and light meromyosin (LMM). The resulting fragments were separated by $NaDodSO_4$/PAGE (37), and the fluorescently labeled peptides were visualized under ultraviolet illumination.

Superprecipitation assays were carried out with unlabeled and labeled myosin. F-actin and myosin filaments were combined (0.05 mg/ml of actin and 0.1 mg/ml of myosin). Superprecipitation was initiated by the addition of ATP (0.1 mM) and was followed by the change in optical density at 550 nm.

Filament Morphology. Synthetic thick filaments, formed by dialyzing unlabeled and fluorochrome-labeled myosin against F buffer (see below), were examined by electron microscopy after negative staining with 1% uranyl acetate. The axial packing of myosin in thick filaments was studied by treating thick filaments with monoclonal anti-LMM antibody, MF20, for 12 hr, followed by negative-stain electron microscopy (38).

Assembly Assays. To monitor assembly, acceptor- and donor-labeled myosin molecules were combined in a 5:1 ratio of acceptor to donor in HS buffer (0.5 M KCl/5 mM $MgCl_2$/5 mM ATP/0.2 mM dithiothreitol/10 mM Tris maleate, pH 6.9). Baseline fluorescence of the mixture was measured for 10 min, and assembly was initiated by dilution to lower ionic strength (F buffer = 0.1 M KCl/5 mM $MgCl_2$/5 mM ATP/0.2 mM dithiothreitol/10 mM Tris maleate, pH 6.9). Filament assembly was continuously monitored in a Perkin–Elmer 650-40 fluorescence spectrophotometer by observing the decrease in donor fluorescence at 470 nm (fluorescence quench). When the assembly reaction reached equilibrium, as evidenced by no further decrease in donor fluorescence, the extent of thick-filament formation was established by protein determination after sedimentation (100,000 × *g* for 15 min), and filament morphology was assessed by negative-stain electron microscopy. All assembly and exchange assays were performed at constant temperature (20°C).

To measure the critical concentration of myosin, FET assembly assays were performed at different concentrations of myosin (0.005–0.5 mg/ml). When complete assembly had occurred, the percentage fluorescence quench at each concentration of myosin was measured. By assuming complete assembly at 0.5 mg/ml as determined by sedimentation, the concentration of myosin that remained unassembled was calculated from the percent fluorescence quench at a particular protein concentration as a proportion of the maximum quench (0.5 mg/ml).

Exchange Assays. Donor- or acceptor-labeled synthetic thick filaments were separately prepared by dialysis against F-buffer. Labeled filaments were then mixed at a 5:1 molar ratio of acceptor to donor, and myosin exchange was monitored by following the quenching of donor fluorescence at 470 nm. Filaments were visualized by negative-stain electron microscopy before and after exchange.

For exchange assays using ^{125}I-myosin, isotope-labeled myosin was prepared by the Bio-Rad Enzymobead procedure with 1 mCi (1 Ci = 37 GBq) of $Na^{125}I$ and 200 μg of myosin. For this assay 2, 5, or 10 μg of unassembled ^{125}I-labeled myosin was added to a 1-ml solution of synthetic thick filaments (1 mg/ml) and incubated for 3 hr. Filaments were sedimented (100,000 × *g* for 15 min), and the extent of myosin exchange was calculated from the specific activities of pelleted thick filaments and unassembled myosin in the supernatant.

All protein determinations were made by the Bradford Assay (39) using albumin standards and by A_{280}.

RESULTS

Characterization of Fluorescently Labeled Myosin. The stoichiometry of fluorochrome binding was established by spectrophotometry (Table 1). Both donor (IAEDANS) and acceptor (IAF) fluorochromes covalently bound to myosin with a dye-to-protein molar ratio of 6–8 fluorochromes per intact myosin molecule. Chymotryptic digests of labeled myosin were performed under high-salt conditions to yield heavy meromyosin (HMM) and light meromyosin (LMM) and under low-salt conditions to generate Rod and S1 fragments. The proteolytic fragments were separated by centrifugation (100,000 × *g* for 30 min) at low ionic strength, and the number of fluorochromes bound per subunit was established by using extinction coefficients of the dyes and protein determination. As indicated in Table 1, labeling occurred along both the rod and head portions of the myosin molecule, even though coupling was performed when myosin was in a polymerized form. Superprecipitation, S1 decoration of actin filaments, and ATPase assays (data not shown) indicate that this fluorochrome-labeled myosin is incapable of shortening and has little or no ATPase activity.

Nevertheless, fluorochrome-labeled myosin molecules were assembly-competent and formed synthetic filaments that were morphologically indistinguishable from those prepared with unlabeled myosin (Fig. 1). After dialysis against F-buffer, virtually all labeled and unlabeled myosin sedimented upon centrifugation (data not shown). To assess the axial packing of myosin in synthetic filaments, control and fluorochrome-labeled myosin filaments were incubated in F buffer containing monoclonal antibody MF20 as described. This antibody has been shown to decorate native thick filaments at 14.5-nm intervals defining the axial packing

Table 1. Fluorescent labeling of myosin

Fluorochrome	Dye/protein ratio				
	Myosin	HMM	LMM	S1 fragment	Rod
IAEDANS (donor)	10	4	6	3	7
IAF (acceptor)	9	4	5	3	6

Myosin fragments were obtained by chymotryptic digestion (see *Materials and Methods*). Under high-salt conditions, digestion yields HMM (head-containing fragment) and LMM (tail-containing fragment). Under low-salt conditions, S1 (head-containing fragment) and Rod (tail-containing fragment) are obtained.

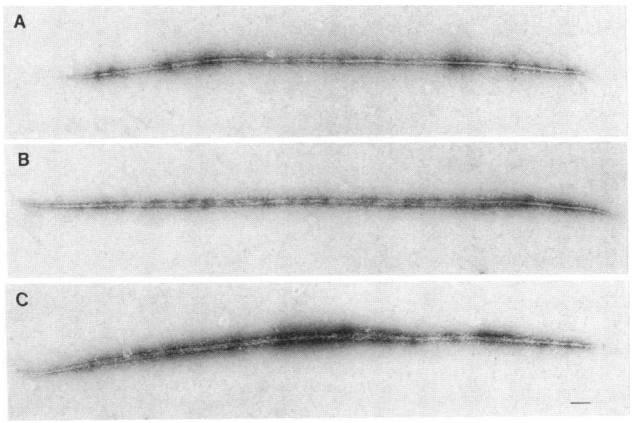

FIG. 1. Morphology of unlabeled and fluorochrome-labeled synthetic thick filaments. (*A*) Unlabeled filaments. (*B*) IAEDANS-labeled filaments. (*C*) IAF-labeled filaments. All were prepared by dialysis against F buffer. For visualization by electron microscopy, synthetic filaments were applied to carbon-coated grids for 15 sec, blotted, rinsed quickly with 0.1 M ammonium acetate (pH 6.8), and negatively stained with 1% uranyl acetate (pH 4.3). (Bar = 100 nm.)

repeat of myosin rods in these structures (38). MF20 decorated unlabeled and labeled synthetic filaments with an identical 14.5 nm periodicity (Fig. 2). Thus, the axial packing of myosin rods in synthetic thick filaments, whether formed from labeled or unlabeled myosin molecules, matched that of native thick filaments. We conclude that fluorochrome labeling of myosin, as described above, does not alter synthetic thick filament structure.

Assembly Assays. Donor- and acceptor-labeled myosin molecules were combined in HS buffer at a 5:1 ratio of acceptor to donor; assembly was initiated by dilution to the lower ionic strength F buffer. Myosin thick-filament formation was monitored continuously by the decrease in donor fluorescence (i.e., the increase in donor quench). After assembly was complete, as evidenced by no further decrease in donor fluorescence, the percentage of fluorescence quench was calculated, and the extent of thick-filament formation was measured by sedimentation and electron microscopy. As shown in Fig. 3, a 43% quench corresponded to complete filament assembly as determined by sedimentation of thick filaments from the assembly mixture.

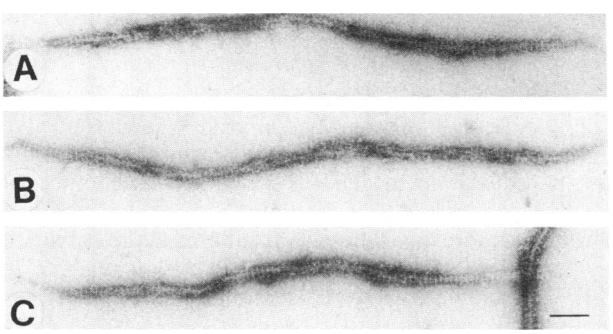

FIG. 2. Axial periodicity of synthetic thick filaments after decoration with monoclonal antibody MF20 (38). Periodicity appears as fine transverse striations (14.5-nm spacing) along the length of the filaments. (*A*) Unlabeled filaments. (*B*) IAEDANS-labeled filaments. (*C*) IAF-labeled filaments. All were prepared by dialysis against F buffer. (Filaments shown in Figs. 1 and 2 are not identical in length, since dialysis leads to a population of filaments that vary in length from 0.5–5 μm.) Filaments were mixed with an equal volume of affinity-purified MF20 (0.04 mg/ml) and incubated overnight at 4°C in F buffer. Filaments were applied to carbon-coated grids for 15 sec, blotted, rinsed quickly with 0.1 M ammonium acetate (pH 6.8), and negatively stained with 1% uranyl acetate. (Bar = 100 nm.)

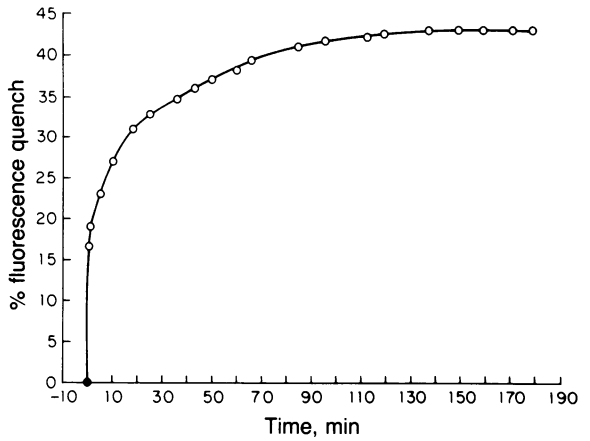

FIG. 3. Detection of myosin thick-filament assembly by fluorescence energy transfer. IAEDANS-labeled myosin (final concentration, 0.1 mg/ml) was combined with IAF-labeled myosin (final concentration, 0.5 mg/ml) in HS buffer; assembly was initiated by reduction of ionic strength by dilution into F buffer and was monitored by the increase in fluorescence quench at 470 nm. When no further changes in fluorescence quench were observed, assembly was determined to be complete by centrifugation (100,000 × *g* for 15 min).

The efficiency of energy transfer during myosin thick-filament assembly under the conditions used here (5:1 ratio of acceptor to donor) was examined by establishing a quench curve. Donor-labeled myosin (0.1 mg/ml) was coassembled with a mixture (0.5 mg/ml) of myosin containing various ratios of acceptor-labeled and unlabeled myosin. The percentage of fluorescence quench was then plotted as a function of the mole fraction of acceptor-labeled myosin within filaments. A linear relationship was observed between the fluorescence quench of donor-labeled myosin and the mole fraction of acceptor-labeled myosin in coassembled thick filaments (Fig. 4). Approximately 40% quench of IAEDANS fluorescence at 470 nm occurred during thick-filament assembly when the mole fraction of IAF-labeled myosin in filaments was 0.8 (i.e., 0.1 mg of IAEDANS-labeled myosin per ml and 0.5 mg of IAF-labeled myosin per ml). This is exactly the fluorescence quench observed in the prior experiment (Fig. 3), indicating that donor (IAEDANS) fluores-

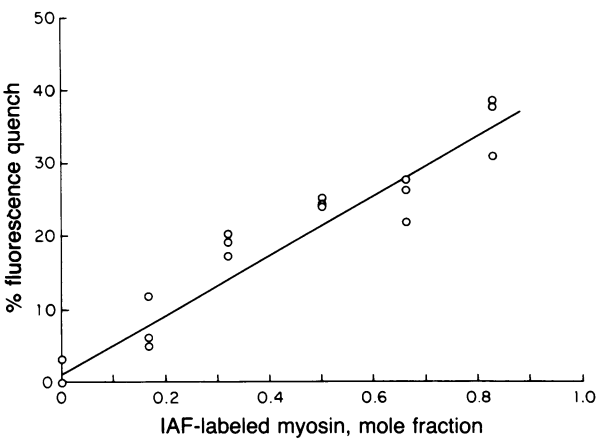

FIG. 4. Efficiency of energy transfer between IAEDANS-labeled myosin and IAF-labeled myosin as a function of the mole fraction of IAF-labeled myosin in thick filaments. Mixtures of IAEDANS-labeled, IAF-labeled, and unlabeled myosin were coassembled by dilution into F buffer. All samples contained a constant amount (0.1 mg/ml) of IAEDANS-labeled myosin and a mixture (0.5 mg/ml) of IAF-labeled and unlabeled myosin containing increasing proportions of IAF-labeled myosin. Percent quench was calculated from the final fluorescence after 3 hr.

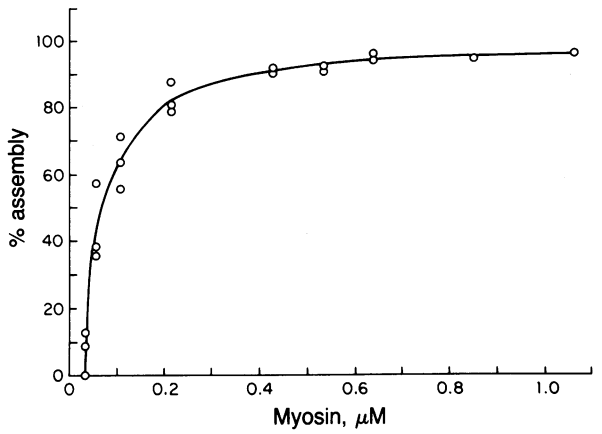

FIG. 5. FET assembly assays performed at different myosin concentrations. IAF-labeled and IAEDANS-labeled myosin were combined in HS buffer in a 5:1 ratio at various total myosin concentrations, and assembly was followed by the increase in donor fluorescence quench after dilution into F buffer. When no further increase in quench was observed, the percentage of assembly was calculated from the percentage of fluorescence quench observed at each myosin concentration.

cence quench measured during assembly is proportional to the mole fraction of acceptor.

Critical Concentration. The critical concentration of myosin—i.e., the concentration that remains unassembled at equilibrium with fully formed filaments under the buffer conditions used here—was measured using FET. The high sensitivity of the FET assay allowed us to perform assembly assays at different myosin concentrations (0.005–1 mg/ml). When complete assembly had occurred, the percentage fluorescence quench (and percentage assembly) at each concentration was measured (Fig. 5). By assuming complete assembly at 0.5 mg/ml as determined by thick filament sedimentation, the concentration of myosin that remained unassembled was calculated at each myosin concentration. The concentration of unassembled myosin in F buffer was 40 nM or 17.2 μg/ml (SD = 3.75) at all concentrations of myosin we examined (Fig. 6).

Exchange of Myosin Between Thick Filaments. The exchange of myosin between thick filaments was examined by mixing acceptor (IAF)-labeled thick filaments with separately prepared donor (IAEDANS)-labeled thick filaments at a 5:1 ratio of acceptor to donor. Within 180 min, 32% quench in donor fluorescence was observed (Fig. 7). Since complete

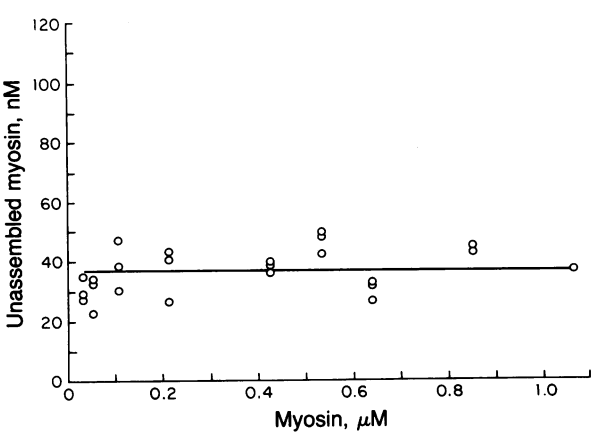

FIG. 6. Calculation of the critical concentration for myosin polymerization. The amount of myosin that remained unassembled at each concentration of myosin was calculated from the data in Fig. 5 (see *Materials and Methods*).

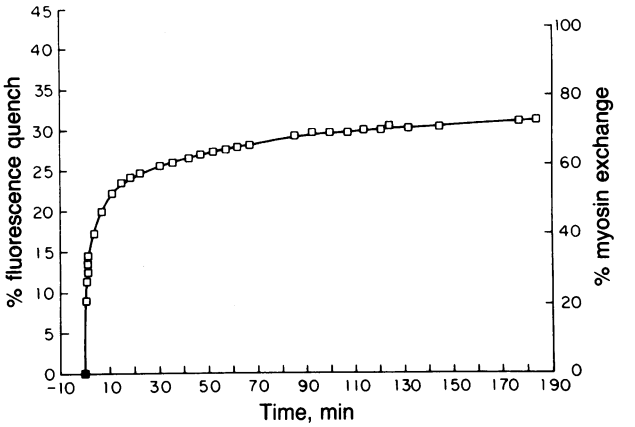

FIG. 7. Exchange of myosin between thick filaments. IAEDANS-labeled synthetic myosin filaments (final concentration, 0.1 mg/ml) were mixed with IAF-labeled filaments (final concentration, 0.5 mg/ml), and exchange of myosin between filaments was monitored by the increase in IAEDANS fluorescence quench at 470 nm.

exchange (i.e., randomization of donor and acceptor myosin) between thick filaments would result in the 43% quench previously observed for filament assembly (see Fig. 3), 32% quench is equivalent to 75% exchange of myosin between thick filaments. We confirmed these data using a different assay system. Subcritical concentrations (2, 5, or 10 μg/ml) of ^{125}I-labeled myosin were added to preformed synthetic thick filaments, and the incorporation of radioisotope-labeled myosin was measured after centrifugation at 100,000 × *g* for 15 min. By comparing the specific activity of sedimented thick filaments with that of unassembled myosin in the supernatant, exchange was calculated to be 81–86% of total myosin (Table 2).

DISCUSSION

To examine thick-filament assembly and myosin exchange, we have established a rapid and simple fluorescence energy transfer (FET) assay. Myosin isolated from adult chicken pectoralis was labeled while in polymerized form with either donor (IAEDANS) or acceptor (IAF) fluorochromes. Although labeling occurred at multiple sites along the molecule, fluorochrome-labeled myosin assembled into synthetic thick filaments of identical length and morphology to those prepared with unlabeled myosin. Furthermore, using an anti-LMM monoclonal antibody, we observed identical packing of labeled and unlabeled myosin into these filaments. The inability of fluorochrome-labeled myosin to superprecipitate and to decorate actin filaments with myosin S1 suggests that these molecules are blocked in their actin interaction and their actin-activated ATPase activity. Nevertheless, our data indicate that these molecules are not altered in their ability to polymerize into thick filaments.

FET assembly assays were used to monitor the polymerization of myosin into thick filaments. A critical concentration for myosin polymerization was 40 nM (17 μg/ml) in 0.1 M KCl, 5 mM $MgCl_2$, 5 mM ATP/0.2 mM dithiothreitol/10

Table 2. Exchange of ^{125}I-labeled myosin into thick filaments

^{125}I-labeled myosin, μg/ml	Soluble myosin		Myosin in filaments		% exchange
	μg/ml	cpm/ml	μg/ml	cpm/ml	
2	18.6	1000	980	47,000	83
5	17.5	3000	980	137,000	81
10	18.0	5000	980	242,000	86

mM Tris maleate, pH 6.9. The concept of a critical concentration of myosin is not a new one. In their pioneering work, Josephs and Harrington have suggested that skeletal muscle myosin filaments exist in equilibrium with monomers (40–42) and that filament assembly follows the Gilbert theory of polymerization (43), which predicts that polymers appear only after a critical concentration required to initiate assembly has been exceeded. Nonmuscle myosin also exhibits a critical concentration (44) that like that of skeletal muscle is dependent on ionic strength, pH, and hydrostatic pressure. A critical concentration for smooth muscle myosin assembly has not been observed (45), for the monomer concentration in equilibrium with filaments of smooth muscle myosin appears to increase with the total myosin concentration. However, the myosin used in those studies was largely unphosphorylated, which could account for the differences observed.

The concept of a critical concentration suggests the existence of a dynamic equilibrium between a small, but constant, pool of unassembled myosin molecules and molecules within fully assembled thick filaments. Using two different assay systems, FET and ^{125}I-labeled myosin incorporation, we observed rapid and extensive exchange of myosin between thick filaments. The extent of myosin exchange *in vivo* and the role of myosin-associated proteins in regulating exchange remains to be established. Nevertheless, these observations suggest a possible mechanism by which myosin isoform transitions during muscle development and myosin turnover in the adult could be mediated through a pool of soluble myosin molecules. Withdrawal of myosin from the equilibrium exchange pool by the action of proteases or myosin-binding proteins might favor thick-filament disassembly. Observations from a number of other laboratories strongly support the concept of exchange reactions for filamentous myosin. In studies with minifilaments, Reisler (46) observed that upon addition of salt, minifilaments reorganize into longer filaments. This process undoubtedly involves a series of dissassembly and reassembly reactions. Pepe and Chowrashi (47) and Pinsett-Harstrom (48) have also demonstrated extensive reorganization of filaments in the absence (47) and presence (47, 48) of ATP.

Although the FET studies presented here were ideally suited to examining the extent of thick filament formation and myosin exchange, elucidation of the exact relationship between the kinetics of the FET reaction and kinetics of assembly or exchange requires further analysis.

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