FLUORESCENCE ENERGY TRANSFER STUDIES OF MYOSIN THICK FILAMENT ASSEMBLY

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Thick myofilaments of striated muscles have been shown to be polymers of myosin associated with several accessory proteins, such as C-protein and M-protein. Myosin tails make up the backbone of the filaments while the myosin heads lie at the filament surface and cross-bridge myosin with actin filaments. The assembly and structure of thick filaments have been extensively studied (1) by a variety of assay systems, including electron microscopy (2), high speed and differential velocity sedimentation equilibrium (3), pressure jump (4), light scattering (5), and electric birefringence (6). Although these studies have provided valuable information on the states of myosin in solution and the overall process of thick filament assembly, knowledge of the mechanisms regulating myosin polymerization and thick filament remodeling in myofibrils remains incomplete. To address this issue a fluorescence energy transfer (FET) assay (7) has been established to examine the assembly of myosin into thick myofilaments and the exchange of myosin between preassembled thick filaments. FET is a simple, sensitive, and physically nonperturbing assay that combines excellent sensitivity for quantification of protein-protein interactions together with continuous observation of the association-dissociation events.

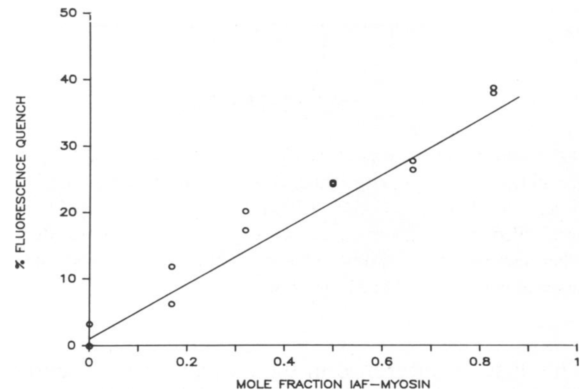

FIGURE 1 Efficiency of energy transfer between IAENS-labeled myosin and IAF-labeled myosin as a function of the mole fraction of IAF-labeled myosin in thick filaments. IAENS-labeled myosin = 0.1 mg/ml. Total myosin = 0.6 mg/ml.

RESULTS

Myosin purified from adult chicken pectoralis muscle was assembled into synthetic thick filaments by dialysis against a low salt assembly buffer (0.1 M KCl, 10 mM potassium phosphate, pH 6.9) and labeled (8) with either 5-(iodoacetamidoethyl)-amino napthalene-1-sulfonic acid (IAENS; donor) or iodoamidofluorescein (IAF; acceptor). Preassembled synthetic thick filaments were labeled to permit fluorochrome binding at sites that do not interfere with the assembly process. Labeled filaments were dialyzed against a high salt buffer (0.5 M KCl, 10 mM potassium phosphate, pH 7.2) to remove unbound fluorochrome and to dissociate filaments. Binding of 9–10 fluorochromes per intact myosin molecule was obtained, and fluorescently labeled myosin was determined to be assembly competent by sedimentation assays and electron microscopy. Limited chymotryptic digests of labeled myosin indicated that the fluorochromes were distributed throughout the myosin molecule (i.e., S1, S2, and LMM).

Fluorochrome-labeled myosin was used to examine assembly into thick filaments. Donor and acceptor labeled myosin were combined in a high salt buffer (0.5 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM DTT, 10 mM Tris-maleate, pH 6.9) that does not allow assembly. Baseline fluorescence was determined for 10 min to insure that no fluorescence changes occurred before assembly and assembly initiated by dilution into a lower ionic strength buffer (0.1 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM DTT, 10 mM Tris-maleate, pH 6.9). Assembly was continuously monitored in a Perkin-Elmer 650-40 fluorescence spectrophotometer by observing the decrease in donor fluorescence at 470 nm. After assembly was complete, as evidenced by no further decreases in donor fluorescence,

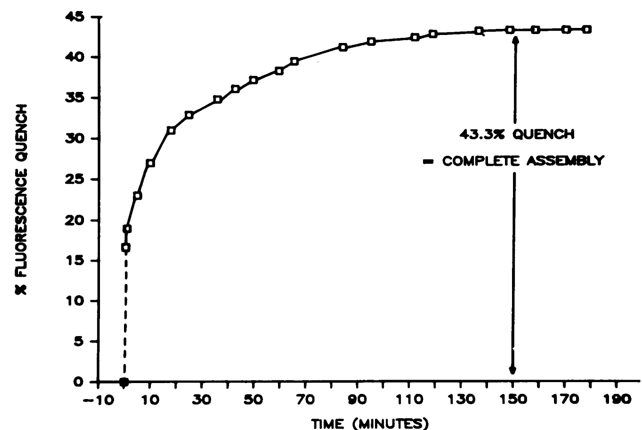

FIGURE 2 Detection of myosin thick filament assembly by fluorescence energy transfer. Coassembly of IAENS-labeled myosin with IAF-labeled myosin monitored by increase in IAENS fluorescence quench at 470 nm.

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percent fluorescence quench was calculated and the extent of thick filament formation measured by sedimentation and electron microscopy. To determine the efficiency of energy transfer in myosin thick filaments, a fluorescence quench curve was obtained (Fig. 1). Donor-labeled myosin was coassembled with a mixture containing various proportions of acceptor-labeled and unlabeled myosin, and percent fluorescence quench plotted as a function of the mole fraction of acceptor-labeled myosin in filaments. The extent of fluorescence quench of donor-labeled myosin is proportional to the mole fraction of acceptor-labeled myosin in coassembled thick filaments. Approximately 40% quench of IAENS fluorescence at 470 nm occurs during thick filament assembly when the mole fraction of IAF-labeled myosin in filaments is 0.8.

The kinetics of myosin polymerization were examined by mixing acceptor-labeled with donor-labeled myosin in a 5:1 ratio and assembly monitored by the decrease in donor fluorescence with time. As shown in Fig. 2, $t_{1/2}$ of assembly was <4 min, and complete assembly was observed at 120 min. 43% quench corresponded to complete assembly as determined by sedimentation of thick filaments from the assembly mixture. The critical concentration of myosin, i.e., the concentration of myosin that remained unassembled at equilibrium with fully formed filaments, was 15–20 μg/ml at a filament concentration of 0.8–1.0 mg/ml.

To detect exchange of myosin between thick filaments, preformed acceptor-labeled thick filaments were mixed in a 5:1 ratio with separately prepared donor-labeled filaments; the resulting change in donor fluorescence was then monitored. Under these conditions, 32% quench in donor fluorescence was observed within 180 min (Fig. 3). Because complete exchange, i.e., total randomization of donor and acceptor-labeled myosin between thick filaments, would result in the 43% quench observed for assembly (Fig. 2), 32% quench is equivalent to 75% exchange of myosin between thick filaments. The $t_{1/2}$ of exchange was <5 min. Sedimentation of filaments after exchange established that myosin remained fully assembled with a critical concentration of 15–20 μg/ml. To determine if myosin exchange occurs between thick filaments and the pool of soluble myosin, a different exchange assay was performed. Trace amounts (5 or 15 μg) of

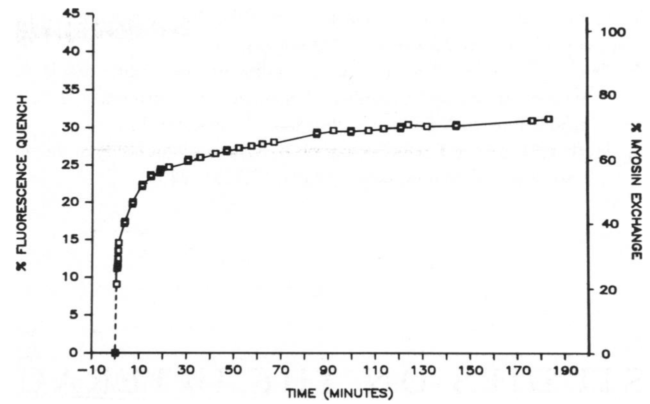

FIGURE 3 Detection of myosin exchange between thick filaments. Exchange between IAENS-labeled filaments and IAF-labeled filaments measured by increase in IAENS fluorescence quench at 470 nm.

^{125}I-myosin was added to 1 ml of a 1 mg/ml solution of synthetic thick filaments and the incorporation of radioisotope-labeled myosin into thick filaments measured by sedimentation. By comparing the specific activity of pelleted thick filaments to that of the unassembled myosin in the supernatant, exchange was calculated to be 81–86% (Table I).

These results indicate that FET is a sensitive and reproducible technique for examining thick filament formation and myosin exchange. By two different assay systems, rapid and extensive exchange of myosin between thick filaments has been observed. Exchange therefore becomes a possible in vivo mechanism for myosin turnover in thick filaments. Detailed kinetic analyses of polymerization and exchange, as well as a determination of the critical concentration of myosin required to spontaneously initiate thick filament formation are now in progess.

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TABLE I
EXCHANGE OF ^{125}I-MYOSIN INTO THICK FILAMENTS

^{125}I-myosin	Soluble Myosin		Myosin in Filaments		Percent Exchange*
ug/ml	*ug/ml*	*CPM/ml*	*μg/ml*	*CPM/ml*	
5	17.5 ± 3	3,000	980 ± 10	137,000	81.0 ± 12
15	18.0 ± 2	5,000	980 ± 10	242,000	86.0 ± 11

$$* = \frac{\text{Sp. Act. myosin in filaments}}{\text{Sp. Act. unassembled myosin}} \times 100.$$

$$= \frac{\text{CPM in filaments/[myosin in filaments]}}{\text{CPM unassembled/[unassembled myosin]}} \times 100.$$

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STUDIES ON THE INTERACTIONS BETWEEN THE SUBUNITS OF SKELETAL MUSCLE TROPONIN USING FLUORESCENCE QUENCHING, PHOTOCHEMICAL CROSS-LINKING, AND EXCITATION ENERGY TRANSFER TECHNIQUES

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The thin filament proteins troponin (Tn) and tropomyosin are responsible for the regulation of skeletal muscle contraction by Ca^{2+}. The first event in this Ca^{2+}-regulation process is the binding of Ca^{2+} to TnC, one of the three subunits of Tn. To understand how this Ca^{2+}-triggering signal is tramsitted from TnC to the other components of the thin filament, we have investigated the interactions between TnC and the other two Tn subunits, TnI and TnT, using three different but complementary techniques. Using fluorescence quenching techniques, we have previously shown that the Stern-Volmer quenching rate constant for TnC labeled at its single sulfhydryl group (Cys-98) with the fluorescence probe 1,5-IAEDANS[1] decreases by a factor of 3.5 when either TnI or TnT are bound. This is consistent with the view that in both the TnC·TnI and the TnC·TnT binary complexes the TnI or TnT moieties bind to TnC near the probe attachment site and thus shield the probe from collision with quenchers in the medium. In the present studies, the proximity relationships between TnC and TnI or TnT were further explored using photochemical cross-linking and fluorescence energy transfer techniques.

MATERIALS AND METHODS

TnC was labeled at Cys-98 with the site-specific photocrosslinker BP-Mal (Molecular Probes, Junction City, OR) as described (2). Irradiation was carried out in a Rayonet RPR-100 "Photochemical Reactor" equipped with 16 "3,500" lamps (Southern New England Ultraviolet, Hamden CT). TnI and TnT were ^{3}H-labeled before cross-linking by reductive methylation (3). Gel bands were cut out and counted as previously described (4). Labeling of TnC at Cys-98 with 1,5-IAEDANS was carried out as described in reference 5. Labeling of TnI at Cys-133 with DAB-Mal (Molecular Probes) was carried out by incubating intact Tn with a twofold molar excess of DAB-Mal at 25° for 2 h in a buffer containing 0.1 M KCl, 0.1 mM $CaCl_2$, 20 mM Hepes, pH 7.5. After dialysis to remove unreacted reagents, the labeled TnI was isolated by urea-DEAE column chromatography. Steady-state fluorometry was carried out on a Perkin-Elmer MPF-4A spectrofluorometer. Fluorescence lifetime measurements were done on a modified Ortec 9,200 ns fluorometer as previously described (6). All experiments were carried out in a buffer containing 0.1 M KCl, 20 mM Hepes, pH 7.5, at 25°.

RESULTS AND DISCUSSION

Our results show that TnI or TnT can be cross-linked to BP-TnC after irradiation of the BP-TnC·TnI or the BP-TnC·TnT binary complex (Fig. 1), confirming the conclusion derived from our fluorescence quenching studies that either subunit binds to TnC in the vicinity of Cys-98. Irradiation of the BP-TnC·TnI.TnT ternary complex produced multiple cross-linking bands (Fig. 1) whose identities were ascertained by using ^{3}H-labeled TnI or TnT initially to form the complex. These studies established that both TnI and TnT are cross-linked to BP-TnC in the ternary complex, suggesting a geometry for the Tn complex in which all three subunits bind near Cys 98 of TnC.

Both steady-state and lifetime measurements revealed that in the ternary complex, substantial energy transfer

[1]*Abbreviations used in this paper:* 1,5-IAEDANS, 5-[2-(iodoacetyl)aminoethyl] aminonaphthalene-1-sulfonic acid; DAB-Mal 4-dimethylaminophenylazophenyl-4-maleimide; BP-Mal, benzophenone-4-maleimide; BP-TnC, TnC labeled with BP-Mal.

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