The Rates of Switching Movement of Troponin T between Three States of Skeletal Muscle Thin Filaments Determined by Fluorescence Resonance Energy Transfer*

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Striated muscle contraction is regulated by troponin (Tn)1 and tropomyosin (Tm) on actin filaments in response to intracellular Ca2+ levels (1, 2). Tm, an extended α-helical coiled-coil dimmer, binds end to end along the actin filament and covers seven actin monomers. Tn is a complex of three proteins: TnC, TnI, and TnT. TnI inhibits actin-myosin interaction, and Tn suppresses the inhibitory effect of TnI by binding Ca2+. TnT binds to Tm and integrates the whole Tn complex into the thin filament. The extended N-terminal domain of TnT interacts with Tm in a Ca2+-independent manner, whereas the C-terminal domain forms globular domain with other troponin subunits (TnI and TnC) and interacts with Tm in a Ca2+-dependent manner (3). The Ca2+-binding to TnC triggers a series of conformational changes of thin filaments, and then the active cross-bridge cycle is turned on (4, 5).

Early explanation for the regulation mechanism is that the skeletal muscle thin filament has two states, “on” and “off” states depending on Ca2+ concentrations. Recent kinetic studies proposed an additional state of the thin filament induced by the strong binding of myosin. Instead of a two-state model based on Ca2+-induced on-off switching, a three-state model has been proposed in which a thin filament exists in rapid equilibrium between three states, “blocked,” “closed,” and “open” (6). The equilibrium between the blocked and closed states is calcium-sensitive, and strong myosin binding stabilizes the open state. Structural studies, such as three-dimensional image reconstructions of electron micrographs and x-ray diffraction, have shown three positions of Tm corresponding to three states of the thin filament (7, 8). However, it is still not clear whether the conformational change observed by both x-ray and three-dimensional image reconstructions of electron micrographs measurements could be entirely attributed to movement of Tm or to a change in the Tn position and/or in actin structure (9). Fluorescence resonance energy transfer (FRET) also provides structural information based on distances between probes attached to specific residues on proteins. This method can easily detect a conformational change in tertiary and quaternary structure of proteins because the transfer efficiency is a function of the inverse of the sixth power of the distance between probes. FRET between probes attached to TnI and actin showed a significant extent of Ca2+-induced and also S1-induced movement of TnI on the reconstituted thin filament (10–14). Not only TnI but also TnT changes positions on the actin filament corresponding to three states of the thin filament (15). The movements of TnI and TnT between these states are impaired on the thin filament reconstituted with a functionally deficient mutant Tm (D234Tm) (14, 16). D234Tm, in which three of seven repeats have been deleted, inhibits actomyosin-MgATPase even in the presence of Ca2+ and Tn (17). The transitions between three positions of TnI and TnT on the thin filament are closely related to the regulation mechanism. Thus, FRET measurements provided a structural evidence for three states of thin filament (14–16). The local dissociation of TnI from actin was demonstrated by use of the excimer fluorescence of pyrene-labeled Tm (18). TnI and TnT-Ca2+ bind to the closed state of actin-Tm, and their binding is greatly weakened in the S1-induced open state.

For better understanding of the regulation mechanism, it is
important to know the transition rates between the three states of thin filaments. Numerous stopped-flow measurements have been carried out to study the kinetics of the conformational change of the thin filament (19–22). In these experiments, fluorescent probes were attached to Tm or actin, and the time course of the fluorescence intensity change was traced for kinetic studies of conformational changes of Tm and actin in response to three states of thin filaments. However, the fluorescence intensity change measured in these experiments indicates some environmental change around the probe, but it does not necessarily mean a spatial rearrangement of thin filaments. On the other hand, the change in the transfer efficiency of FRET indicates a spatial rearrangement of thin filaments. The change in the transfer efficiency of FRET between probes attached to TnT and actin was monitored by donor fluorescence intensity to determine the rates of Ca\(^{2+}\) - and S1-induced movements of TnT on the reconstituted thin filament (23, 24). In the present study, the rates of Ca\(^{2+}\) - and S1-induced movements of TnT were determined by measuring FRET between probes attached to Cys-374 of actin and Cys-60 or Cys-250 of the point-mutated TnT25k fragment on the reconstituted thin filaments. Cys-60 is located on the tropinin tail domain, and Cys-250 is on the globular domain. As compared with the rate constants of S1 binding or dissociation determined by light scattering measurements, these measurements provide a better understanding for a switching mechanism by Tn in striated muscle regulation.

MATERIALS AND METHODS

Reagents—Phalloidin from Amanita phalloides was purchased from Sigma. IAEDANS and DABMI were purchased from Molecular Probes. All other chemicals were of analytical grade.

Proteins—Actin, S1, and Tn were prepared from rabbit skeletal muscle as reported previously (10). TnT was extracted from rabbit hearts as reported previously (10). Single cysteine TnT25k mutants (E60C and S250C) were prepared as reported previously (15). Actin was labeled at Cys-374 with DABMI as mentioned previously (14). The Tn complex labeled at Cys-133 of TnT with AEDANS (AEADANS-I133-Tn) was prepared as reported previously (14). TnT25k mutants were labeled with IAEDANS, and reconstitution of ternary Tn complexes with AEADANS-labeled TnT25k mutants (AEADANS-T60-Tn and AEADANS-T250-Tn) was carried out as reported previously (15). Cys-60 is located at the second residue from the N terminus, and Cys-250 is located at the seventeenth residue from the C terminus of the 25-kDa fragment of TnT. ATPase activity measurements indicated that Tn complexes reconstituted with the AEADANS-labeled TnT25k mutants retained the regulatory activity as well as the wild-type Tn (15). For stopped-flow measurements, equimolar phalloidin to actin was added to stabilize the actin filament.

Spectroscopy—Measurements—Absorption was measured with a Hitachi U-3310 spectrophotometer. Steady state fluorescence was measured with a PerkinElmer LS50B fluorometer. Protein concentrations were determined by use of absorption coefficients of \(A_{280 \text{ nm}} = 0.63 \ (\text{mg/ml})^{-1} \ \text{cm}^{-1}\) for G-actin, \(A_{285 \text{ nm}} = 0.24\) for Tm, 0.45 for Tn, and 0.67 \(\ (\text{mg/ml})^{-1} \ \text{cm}^{-1}\) for TnT25k. The concentration of labeled protein was measured with the BCA protein assay reagent by using the nonlabeled protein. Relative molecular masses of 42 kDa for actin, 66 kDa for Tm, and 64 kDa for reconstituted TnT with TnT25k mutants were used. The absorption coefficients of 24,800 \(\ (\text{m}^{-1} \ \text{cm}^{-1})\) at 460 nm for DABMI (25) and 6100 \(\ (\text{m}^{-1} \ \text{cm}^{-1})\) at 337 nm for IAEDANS (26) were used to determine the labeling ratios. The typical labeling ratios were 1.0 for DAB-F-actin, 0.75 for AEADANS-T250-Tn, and 0.74 for AEADANS-T60-Tn. pCa values were calculated from the concentrations of added EGTA and CaCl\(_2\) using the numerical constants of Schwarzbench et al. (27).

Stopped-flow Measurements—Kinetic measurements were performed using an Applied Photophysics Ltd. Model SX.18 MV stopped-flow spectrofluorometer. The excitation monochromator was set at 340 nm for fluorescein or 500 nm for the light scattering measurements, and the light from 420–540 nm was collected through a broad band cut filter (Chroma Technology Co.) placed at the emission side. The instrumental dead time was 1.63 ms under the present experimental conditions. For each experiment, the reaction curves are the average of 10 reaction traces. The data were fitted with a nonlinear least-squares procedure to a single or a double exponential expression from which the rate constants were calculated. Prior to the stopped-flow experiments, the pH of the protein sample solutions were adjusted to the experimental values, and the pH of the mixing buffer solutions (Ca\(^{2+}\) or EGTA solutions) was adjusted by KOH to give the values of the experimental pH after rapid mixing with the protein solutions. Data sets were fit to the single and double exponential equation with a floating end point using the Applied Photophysics Ltd. SX.18MV kinetic spectrometer work station software and Microsoft Excel.

RESULTS

\(\text{Ca}^{2+}\)-dependent Conformational Change—In the present study, IAEDANS attached to Cys-60 or Cys-250 of TnT was used as the FRET donor, and DABMI attached to Cys-374 of actin was as the energy acceptor. From steady-state fluorescence measurements, we reported previously that a large difference in the transfer efficiency between these donor and acceptor probes was observed between the presence and absence of \(\text{Ca}^{2+}\) (15). The change in the transfer efficiency upon \(\text{Ca}^{2+}\) binding corresponds to the increase in the distance by 3.6 A for AEADANS-T60-Tn and 7 A for AEADANS-T250-Tn (15). This \(\text{Ca}^{2+}\)-dependent conformational change was measured by changing the free \(\text{Ca}^{2+}\) concentrations. Fig. 1 shows the fluorescence titration curves of AEADANS-T250-Tn/Tm/DAB-F-actin (in the presence of the acceptor) and AEADANS-T250-Tn/Tm/F-actin (in the absence of the acceptor) as a function of the free \(\text{Ca}^{2+}\) concentration. The curve in the presence of the acceptor (FRET) shows a large increase in the donor fluorescence with increasing free \(\text{Ca}^{2+}\) concentration, whereas the curve in the absence of acceptor shows a decrease in the donor fluorescence. The curve of FRET shows a very sharp transition with a midpoint at pCa 6.6. The results indicate that the rearrangement of the spatial relationship between TnT and actin in reconstituted thin filaments occurs with a highly cooperative mode. The change in FRET between probes attached to TnT and actin directly reflects the change of the physiological conditions of the reconstituted thin filament (i.e. active and inhibitory states).

Kinetics of \(\text{Ca}^{2+}\)-induced Movement of TnT—FRET in combination with the stopped-flow method (time-resolved FRET) was used to measure the \(\text{Ca}^{2+}\)-induced movement of TnT by rapidly changing the free \(\text{Ca}^{2+}\) concentration. The change in the transfer efficiency after changing the solvent conditions from \(\text{Ca}^{2+}\) to \(\text{Ca}^{2+}\) (or vice versa) was monitored by the donor-fluorescence intensity in the presence (Fig. 2, A and C)
and absence (Fig. 2, B and D) of the acceptor. The reconstituted thin filament in 30 mM KCl, 0.1 mM ATP, 2 mM MgCl₂, 1 mM NaNO₃, and 20 mM MOPS or 20 mM MES (buffer A) with 0.5 mM EGTA (buffer A-EGTA) or 0.1 mM CaCl₂ (buffer A-Ca) was mixed with the same volume of buffer A containing 4 mM CaCl₂ or 3.75 mM EGTA, respectively. Fig. 2, A and C, shows the time courses of the fluorescence intensity of AEDANS-T250-Tn/Tm/DAB-F-actin after changing Ca²⁺ concentrations at pH 7.0. From −Ca²⁺ to +Ca²⁺ (Fig. 2A), the fluorescence intensity increased (the transfer efficiency decreased) very rapidly and reached a final fluorescence level within 10 ms after the flow-stop point. The observed fluorescence intensity change was analyzed by a single exponential process with the rate constant of 376 ± 56 s⁻¹. From +Ca²⁺ to −Ca²⁺ (Fig. 2C) the fluorescence intensity decreased with the rate constant of 91.0 ± 14 s⁻¹. On the other hand, Fig. 2, B and D, shows the time courses of the fluorescence intensity of AEDANS-T250-Tn/Tm/F-actin (in the absence of the acceptor) after changing Ca²⁺ concentrations at pH 7.0. Contrary to the case in the presence of the acceptor, the fluorescence intensity decreased with the rate constant of 345 ± 38 s⁻¹ from −Ca²⁺ to +Ca²⁺ (Fig. 2B) and increased with the rate constant of 85.9 ± 10 s⁻¹ from +Ca²⁺ to −Ca²⁺ (Fig. 2D).

The time courses of fluorescence intensity changes were followed at pH 7.5, 6.5, and 6.0. From −Ca²⁺ to +Ca²⁺, the fluorescence intensity of AEDANS-T250-Tn/Tm/DAB-F-actin increased with rate constants of 641 ± 96 s⁻¹ at pH 7.5, 208 ± 31 s⁻¹ at pH 6.5, and 151 ± 23 s⁻¹ at pH 6.0. On the other hand, from +Ca²⁺ to −Ca²⁺ the fluorescence intensity of AEDANS-T250-Tn/Tm/DAB-F-actin decreased with rate constants of 83.1 ± 12 s⁻¹ at pH 7.5, 109 ± 16 s⁻¹ at pH 6.5, and 150 ± 23 s⁻¹ at pH 6.0. The rate constants of Ca²⁺-induced TnT movement strongly depended on the pH. As the pH decreased, the transition rate from −Ca²⁺ to +Ca²⁺ became slower, but the reverse rate became faster. The same measurements using AEDANS-T60-Tn and AEDANS-I133-Tn were carried out at pH 7.5, 7.0, 6.5, and 6.0. The results were summarized in Fig. 3.

**Kinetics of S1-induced Movement of TnT**—According to the three-state model of thin filaments, rigor S1 binding to the regulated thin filament induces the open state (6). Steady-state FRET measurements showed that the transfer efficiency between probes attached to Cys-60 or Cys-250 of the TnT25k mutant and Cys-374 or Gln-41 of actin in the reconstituted thin filament decreases significantly by rigor S1 binding (15). In the absence of the acceptor, the donor fluorescence did not change appreciably by rigor S1 binding. The change in the transfer efficiency was monitored by the donor-fluorescence intensity in the presence of the acceptor. Using AEDANS-T60-Tn and DAB-F-actin, time courses of the light scattering and fluorescence intensity changes of the reconstituted thin filament were followed after S1 binding or dissociation in the presence of Ca²⁺ (transition from the closed to open states or vice versa) (Fig. 4). In the case of S1 dissociation, the reconstituted thin filaments and S1 complex in 30 mM KCl, 2 mM MgCl₂, 20 mM MOPS, pH 7.5, and 1 mM NaNO₃ (buffer F) with 0.1 mM CaCl₂ (buffer F-Ca) was mixed with the same volume of buffer F-Ca including 1.0 mM ATP at 20 °C. The light scattering intensity decreased very rapidly and reached the final level at −4 ms after the flow-stop point. The observed light scattering intensity change was only
The Rates of Troponin T Movement

Fig. 3. pH dependence of the rate constants of Ca$^{2+}$-induced conformational change. Using AEDANS-I133-Tn (circle), AEDANS-T60-Tn (triangle), and AEDANS-T250-Tn (diamond), the same experiments as in Fig. 2 were carried out at pH 6.0, 6.5, 7.0, and 7.5. Closed and open symbols represent the rate constants of transition from $-\text{Ca}^{2+}$ to $+\text{Ca}^{2+}$ and from $+\text{Ca}^{2+}$ to $-\text{Ca}^{2+}$, respectively. MOPS for pH 7.5 and pH 7.0 and MES for pH 6.5 and pH 6.0 were used.

−20% of the total change under the present experimental conditions. The whole process of the light scattering change was regarded as a single exponential process and fitted with the rate constant of 975 ± 107 s$^{-1}$ (Fig. 4, A (1)), although such fast rate is near the limitation of the present instrumental conditions (dead time of 1.63 ms). On the other hand, the fluorescence intensity decreased much slower than the light scattering intensity (Fig. 4, A (2)), and the trace was analyzed by a single exponential curve with the rate constant of 372 ± 48 s$^{-1}$.

In the case of S1 binding, the reconstituted thin filament in 140 mM KCl, 2 mM MgCl$_2$, 20 mM MOPS, pH 7.5, and 1 mM Na$_2$ATP (buffer F') with 0.1 mM CaCl$_2$ (buffer F$'_+\text{Ca}$) was mixed with the same volume of S1 in buffer F$'_+\text{Ca}$ at 20 °C. When S1 was mixed with thin filaments, the fluorescence intensity increased slightly faster than the light scattering intensity (Fig. 4B). The curves were analyzed by a single exponential process with the rate constants of 10.3 ± 1.3 s$^{-1}$ for light scattering (curve 1) and 12.2 ± 1.6 s$^{-1}$ for fluorescence (curve 2).

Time courses of the light scattering and fluorescence intensity changes of the reconstituted thin filament after S1 binding or dissociation were followed also in the absence of Ca$^{2+}$ (transition from the relaxed to open states or vice versa). In the case of S1 dissociation (Fig. 5A), the reconstituted thin filament and S1 complex in buffer F with 1.0 mM EGTA (buffer F$'_-\text{Ca}$) was mixed with the same volume of buffer F$'_-\text{Ca}$ including 1.0 mM ATP at 20 °C. The light scattering intensity decreased as rapidly as the case in the presence of Ca$^{2+}$, and the trace was fitted by a single exponential process with the rate constant of 888 ± 98 s$^{-1}$ (Fig. 5A (1)). On the other hand, the fluorescence intensity (Fig. 5A (2)) decreased much slower than the light scattering intensity, and the curve was analyzed by a single exponential process with the rate constant of 407 ± 53 s$^{-1}$.

In the case of S1 binding, the reconstituted thin filament in buffer F$'_+\text{Ca}$ with 1.0 mM EGTA (buffer F$'_-\text{Ca}$) was mixed with the same volume of S1 in buffer F$'_-\text{Ca}$ at 20 °C. When S1 was mixed with thin filaments, the fluorescence intensity increased slightly faster than the light scattering intensity (Fig. 5B). The curves were fitted by a single exponential process with the rate constants of 4.5 ± 0.6 s$^{-1}$ for light scattering (curve 1) and 5.9 ± 0.7 s$^{-1}$ for fluorescence (curve 2). The values were approximately half of the rate constants in the presence of Ca$^{2+}$.

Using AEDANS-T250-Tn and DAB-F-actin, time courses of the light scattering and fluorescence intensity change of the reconstituted thin filaments after S1 binding and dissociation were also followed under the same experimental conditions as AEDANS-T60-Tn. Almost the same results were obtained. Results are summarized in Table I.

Effects of ATP Concentrations on Rate Constants—Using AEDANS-T60-Tn, rate constants of the light scattering and fluorescence intensity changes of the reconstituted thin filament after S1 dissociation were determined at various ATP concentrations. Stopped-flow measurements were carried out under the same experimental conditions as shown in Figs. 4A and 5A except for the concentration of ATP. Time courses of the light scattering and fluorescence intensity changes were analyzed with a single exponential process both in the presence and absence of Ca$^{2+}$. Fig. 6 shows the rate constant versus the ATP concentration in the presence and absence of Ca$^{2+}$. The rate constant of the light scattering intensity change (dissociation of S1 from the thin filament) increased markedly as the ATP concentration increased up to 0.5 mM, irrespective of the presence or absence of Ca$^{2+}$. On the other hand, the rate constant of the fluorescence intensity change increased as the ATP concentration increased up to 0.25 mM but did not change at more than 0.25 mM ATP.

DISCUSSION

Using time-resolved FRET between probes attached to Cys-374 of actin and positions 1, 133, or 181 of TnI, we measured the switching rates of TnI between the three states of thin filaments (24). In the present study, probes were attached to positions 60 and 250 on TnT25k fragment and Cys-374 on actin, and time-resolved FRET was measured to determine the switching rates of TnT between the three states of thin filaments.

Ca$^{2+}$-induced Movement of TnT—In contrast to the case of AEDANS attached to positions 1 and 181 on TnI (24), the fluorescence intensity of the donor, AEDANS, attached to positions 60 or 250 on TnT was sensitive to changes in Ca$^{2+}$ concentration. Upon Ca$^{2+}$ binding, the donor fluorescence intensity decreased by 9% for T60-Tn and 14% for T250-Tn in the absence of the acceptor, whereas the fluorescence intensity in the presence of the acceptor increased by 29% for T60-Tn and 40% for T250-Tn under the present experimental conditions. The environmental sensitivity of the donor fluorescence would affect the transition rates measured by time-resolved FRET. Dong et al. (28) developed a method for such correction by converting the fluorescence intensity traces in the presence and absence of acceptor to a FRET efficiency trace. In the present study, the extents of the change of the donor fluorescence in the absence of the acceptor were almost 9 and 14%. Nevertheless, both rate constants in the presence and absence of acceptor were almost the same to each other (376 and 345 s$^{-1}$, Fig. 2, A and B; 91 and 86 s$^{-1}$, Fig. 2, C and D) so that the environmental sensitivity of the donor fluorescence did not seriously affect the calculation of the rate constants of the FRET trace. Indeed, such correction changed the transition rate by <10%.

The transition rate of TnT upon Ca$^{2+}$ binding or detaching was almost the same as that of TnI at pH 7.0, which suggests that the troponin complex moves as a whole on the actin filament with this rate constant. Ca$^{2+}$ binding triggers a series of conformational changes in the Tn complex on the thin filament: (1) the opening of the TnC N domain and the exposure of the hydrophobic patch of the N lobe of TnC, (2) the release of the amphiphilic helix (residues 128–148 in skeletal TnI) from the actin/Tm, (3) the binding of the regulatory region of TnI to TnC, (4) the binding of the C-terminal region of TnT to Tm, and (5) movement of the N-terminal region of TnT. In the Ca$^{2+}$-induced conformational change of the thin filament, the series of spatial rearrangements in the Tn complex would occur without any delay in each step, i.e., synchronously. Therefore, the rate constants of TnI and TnT movements are similar to each other.
Using a mutant TnC (F29W) and dansylaziridine-labeled TnC, Johnson et al. (29) determined the rates of Ca\(^{2+}\)/H\(_{11001}\) binding to and dissociation from the Ca\(^{2+}\)/H\(_{11001}\)-specific site of TnC. Ca\(^{2+}\) dissociates from the Ca\(^{2+}\)/H\(_{11001}\)-specific site with a rate constant of 483 s\(^{-1}\). The rate of Ca\(^{2+}\)/H\(_{11001}\) association with TnC (F29W) increased linearly as the free Ca\(^{2+}\) concentration increased (up to 750 s\(^{-1}\) at 5.1 M Ca\(^{2+}\)), indicating that Ca\(^{2+}\) binds as rapidly as it can diffuse to the protein (29). The rapid fluorescence intensity change of the mutant TnC (F29W) reflects the environmental change of the donor, which may come from the Ca\(^{2+}\) binding itself or from the first event (1), opening of the TnC N domain and the exposure of the hydrophobic patch of the N lobe of TnC. After some time lag, the subsequent spatial rearrangements of the Tn subunits (from (2) to (5)) follow synchronously. Thus, the information of Ca\(^{2+}\) binding triggers a synchronous conformational rearrangement of the Tn subunits, and consequently the Tn complex moves as a whole from the position of the relaxed state to the position of the closed state with a rate constant of ~450 s\(^{-1}\). On the other hand, upon deactivation, Ca\(^{2+}\) detaching triggers a series of conformational changes in the Tn complex on the thin filament: (1) TnC N domain closing, (2) separation of TnI from TnC in the central helix region of the TnC, (3) the binding of the amphiphilic helix of TnI to the actin/Tm, and (4) movement of the TnT to the position of the closed state. Dong et al. (28) measured time-resolved FRET between TnI and TnC to determine the rate of the separation of the regulatory region of TnI from TnC. The rate was determined to be 236 s\(^{-1}\) in the TnI-TnC binary complex and 133 s\(^{-1}\) in the Tn complex. This rate, if measured on the reconstituted thin filament, may be close to the rate of the TnT movement, ~85 s\(^{-1}\). The information of Ca\(^{2+}\) detaching triggers a synchronous conformational rearrangement of the Tn subunits, and consequently the Tn complex moves as a whole to the position of the relaxed state on the thin filament with a rate constant of ~85 s\(^{-1}\) . These rates of the Tn movement on the thin filament by the Ca\(^{2+}\) association and dissociation are rapid enough to account for the speed of skeletal muscle contraction and relaxation.

**pH Dependence**—It is well known that the Ca\(^{2+}\) sensitivity of contraction of skinned fibers prepared from mammalian stri-
ated muscle is reduced by acidic pH and that troponin is responsible for this acidosis. Lowering pH reduces the affinity of skeletal TnC for Ca\(^{2+}\) binding. The pH effect on the Ca\(^{2+}\) affinity of TnC was further modified through other troponin subunits (30). The roles of subunits in the pH dependence on the Ca\(^{2+}\) sensitivity have been extensively studied by exchanging troponin subunits with the isofoms in permeabilized muscle fibers (31, 32). To obtain further insights into the molecular mechanism determining the pH sensitivity of Ca\(^{2+}\) regulation, the pH effects on the transition rates of TnI and TnT upon Ca\(^{2+}\) binding to TnC were examined. The transition rate of TnT movement upon Ca\(^{2+}\) binding decreased significantly as the pH decreased, whereas the rate of TnI movement did not change much (Fig. 3). On the other hand, after Ca\(^{2+}\) detaching from TnC, the both transition rates of TnT and TnI movements increased appreciably as the pH decreased. The switching rate of TnT upon Ca\(^{2+}\) binding to TnC was affected more than that of TnI by lowering pH, suggesting that the synchronous conformational change in the Tn subunits triggered by Ca\(^{2+}\) binding is impaired. On the other hand, a deletion mutant Tm (D234Tm) in which internal actin-binding pseudo-repeats 2, 3, and 4 are missing inhibits the thin filament-activated myosin-ATPase activity whether Ca\(^{2+}\) is present or not (17). The Ca\(^{2+}\)-induced movement of TnT was impaired on this functionary deficient mutant Tm, although the Ca\(^{2+}\)-induced movement of TnI was not affected (14, 16). These indicate that a synchronous movement of the Tn subunits would be critical for the regulation mechanism.

The C-terminal region of TnT forms a globular portion of the Tn complex with TnC and TnI, which is located near residues 150–180 on Tm (33). The C-terminal 17 residues of TnT contain the Tm-binding site that is critical for the Ca\(^{2+}\)-sensitizing activity of TnT (3). This region in which there are no acidic but six basic residues is highly positive. Therefore, the ionic interaction between the Ca\(^{2+}\)-sensitizing region of TnT and Tm would strongly depend on pH. On the other hand, the Ca\(^{2+}\) binding induces the exposure of the hydrophobic region in TnC, which binds the regulatory region of TnI. Thus unlike TnT the Ca\(^{2+}\)-induced movement of TnI would not be affected strongly by lowering the pH.

**S1-Induced Movement of TnT**—Recently, we determined the rates of S1-induced TnI movement by time-resolved FRET (24). Here the transition rates of S1-induced TnT movement were determined. When strongly bound S1 is abruptly dissociated from the thin filament, the transition from the open to closed and relaxed states would occur in the presence and absence of Ca\(^{2+}\), respectively. The change in the light scattering intensity indicates the dissociation of S1 from the regulated thin filament. The rate of dissociation increased as the concentration of ATP increased (Fig. 6). At 0.5 mM ATP, it was >800 s\(^{-1}\). On the other hand, the change in the fluorescence intensity of the donor in the presence of the acceptor (TnT movement) was analyzed by a single exponential curve with a rate constant of ~400 s\(^{-1}\) both in the presence and absence of Ca\(^{2+}\). This rate constant also depends on the ATP concentration as in the case of TnI movement (24). It increased as the concentration of ATP increased up to 0.25 mM but became almost constant over the 0.25 mM ATP concentration. In a previous study (24), the transition curve of TnI movement in the presence of Ca\(^{2+}\) was fitted by a single exponential curve with the rate constant of ~300 s\(^{-1}\), whereas in the absence of Ca\(^{2+}\) the curve was fitted by a double exponential curve with the rate constants of ~400 and ~50 s\(^{-1}\). These indicate that the transition of TnT from the open to relaxed states occurs directly, whereas the transition of TnI movement occurs through an intermediate state (the closed state). That is, TnT moves differentially with TnI. The differential movement suggests a flexible joint between TnI and TnT subunits in the Tn complex.

When S1 binds to the thin filament, the transition to the open state from the closed and relaxed states would occur in the presence and absence of Ca\(^{2+}\), respectively. The light scattering measurements showed that the S1 binding occurred with the rate constant of ~10 and ~5 s\(^{-1}\) in the presence and absence of Ca\(^{2+}\), respectively, in accordance with the previous report (19). Time-resolved FRET showed that the movement of AEDANS-T60-Tn on thin filaments occurred with a slightly higher rate than that observed from the light scattering change in the presence or absence of Ca\(^{2+}\). On the other hand, the movement of TnI occurred with almost the same and slightly slower rates than those observed from the light scattering change in the presence and absence of Ca\(^{2+}\), respectively (24). The results indicate that the S1-induced movement of TnT occurs in more cooperative manner than that of TnI because TnT is an elongated molecule along the actin filament.

**Kinetics with the Fluorescence Signal from Pyrene Probe Attached to Tm**—Geeves and Lehrer (19) measured the rates of transition between the three states of the thin filament by monitoring the fluorescence intensity from the pyrene label attached to Tm. Although the FRET efficiencies in TnI and TnT movements correspond well to the three states of the thin filament, the excimer fluorescence of pyrene attached to Tm is sensitive to S1-induced but not to Ca\(^{2+}\)-induced conformational changes of the thin filament. In the case of Ca\(^{2+}\)-induced dissociation of S1 from the thin filament, the light scattering signals showed the single exponential transients with the rates depending on the ATP concentration linearly. The rates were almost the same as our previous (24) and present results. However, the fluorescence signal from pyrene-labeled Tm showed significant lags irrespective of Ca\(^{2+}\) concentrations. In the case of S1 binding, the light scattering signals showed

| Donor site of Tn | S1 dissociation | S1 binding |
|-----------------|----------------|------------|
|                 | \(k_{LS}\)      | \(k_{FL}\)  | \(h_{LS}\)  | \(h_{FL}\)  |
| TnT E60C        | 975 ± 107      | 372 ± 48   | 10.3 ± 1.3  | 12.2 ± 1.6  |
| TnT S250C       | 939 ± 103      | 391 ± 51   | 11.1 ± 1.3  | 11.1 ± 1.4  |
| TnT E60C        | 888 ± 98       | 407 ± 53   | 4.5 ± 0.6   | 5.9 ± 0.7   |
| TnT S250C       | 828 ± 98       | 440 ± 57   | 5.7 ± 0.7   | 4.9 ± 0.6   |
almost the same results as our previous and present results. However, the fluorescence signal showed the transitions one order faster than those of the light scattering change. They explained the results by a highly cooperative conformational change of thin filaments in which a single S1 binding is sufficient to switch on the cooperative unit of 10–12 actin subunits. Consequently, in the case of ATP-induced dissociation of S1, the lag of the fluorescence signal was observed, and in the case of S1 binding the rapid change in the pyrene fluorescence of Tm was seen. TnT is an elongated molecule of which the N-terminal region extends along the C-terminal region of Tm to the beginning of the next Tm on the actin filament. However, the FRET signal of TnT gave different aspects from the fluorescence signal of the pyrene label attached to Tm. The movement of TnT did not show the similar lag in the case of S1 dissociation nor the rapid transition rate one order faster than the light scattering signal in the case of S1 binding. Here it should be noted that the change in the fluorescence signal from pyrene-labeled Tm shows a conformational change around the environment of the probe but does not necessarily mean the spatial rearrangement of the thin filament. The binding of S1 induces a cooperative conformational change of actin subunits through Tm, and the fluorescence intensity of pyrene on Tm increased faster than the scattering intensity. This conformational change would induce the movement of the N-terminal region of TnT following a further movement of the globular region of the Tn complex. On the other hand, in the case of the ATP-induced dissociation of S1, ATP binds to S1 as rapidly as it can diffuse to the protein, and the number of strongly bound S1 decreases instantaneously. Consequently, S1 dissociation, TnI, and TnT movements occur without the lag. Then, the environmental change around the pyrene probe attached to Tm follows with a lag compared with S1 dissociation and TnT movement.

Steady-state FRET measurements showed the structural evidence for the three states of thin filaments in which Tn changes the positions on the actin filament in response to the three states. Time-resolved FRET measurements determined the rates of movement of TnI and TnT on the transition between the three states of the thin filament. In most but not all aspects, the troponin tail and globular domains move with very similar rates on the transition. That is, a series of conformational changes in the troponin complex during the switching on or off process occurs synchronously. The rates are fast enough to allow the Tn movement to be directly involved in muscle regulation. The crystal structure of the core domain of Tn has been revealed (34). Based on the atomic structures of Tn and actin (35, 36), more studies are necessary to clarify all of the structural changes that occur on the thin filament in combination with the switching movement of the Tn complex. It is especially important to know how the conformational change of actin subunits is related to the movement of the Tn complex.

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REFERENCES
1. Ebashi, S., Endo, M., and Ohtsuki, I. (1969) Q. Rev. Biophys. 2, 351–384
2. Ohtsuki, I., Maruyama, K., and Ebashi, S. (1986) Adv. Protein Chem. 38, 1–67
3. Onoyama, Y., and Ohtsuki, I. (1986) J. Biochem. (Tokyo) 100, 517–519
4. Farah, C. S., and Reinach, F. C. (1995) FEBS Lett. 39, 755–767
5. Gordon, A. M., Homsher, E., and Regnier, M. (2000) Physiol. Rev. 80, 853–924
6. Mckillop, D. F., and Geeves, M. A. (1993) Biophys. J. 65, 693–701
7. Holmes, K. C. (1995) Biophys. J. 68, suppl. 2–7
8. Vibert, P., Craig, R., and Lehmann, W. (1997) J. Mol. Biol. 266, 8–14
9. Squire, J. M., and Morris, E. P. (1998) FASEB J. 12, 761–771
10. Miki, M. (1990) Eur. J. Biochem. 187, 155–162
11. Tao, T., Geng, B. J., and Leavis, P. C. (1996) Science 274, 1339–1341
12. Miki, K., Kobayashi, T., Kimura, H., Hagiwara, A., Hiai, H., and Maeda, Y. (1998) J. Biochem. (Tokyo) 123, 324–331
13. Kobayashi, T., Kobayashi, M., and Collins, J. H. (2001) Biochim. Biophys. Acta 1504, 148–154
14. Hai, H., Sano, K., Maeda, K., Maeda, Y., and Miki, M. (2002) J. Biochem. (Tokyo) 131, 407–418
15. Kimura, C., Maeda, K., Maeda, Y., and Miki, M. (2002) J. Biochem. (Tokyo) 132, 93–102
16. Kimura, C., Maeda, K., Hai, H., and Miki, M. (2002) J. Biochem. (Tokyo) 132, 345–352
17. Landis, C. A., Bohkova, A., Homsher, E., and Tobacman, L. S. (1997) J. Biol. Chem. 272, 14051–14056
18. Zhou, X., Morris, E. P., and Lehrer, S. S. (2000) Biochemistry 39, 1128–1132
19. Geeves, M. A., and Lehrer, S. S. (1994) Biophys. J. 67, 273–282
20. Maytum, K., Lehrer, S. S., and Geeves, M. A. (1998) Biochemistry 19, 1102–1110
21. Maytum, R., Geeves, M. A., and Lehrer, S. S. (2002) J. Biol. Chem. 277, 29774–29780
22. Restar, A. M., Stephens, J. M., and Chalovich, J. M. (2002) Biochemistry 41, 1039–1049
23. Miki, K., and Ito, T. (1983) J. Biol. Chem. 258, 7101–7106
24. Shibata, Y., Kimura, C., Ito, T., and Miki, M. (2004) Biochemistry 43, 10739–10747
25. Lamkin, M., Tao, T., and Lehrer, S. S. (1983) Biochemistry 22, 3053–3058
26. Hudson, E. N., and Weber, G. (1973) Biochemistry 12, 4154–4161
27. Schwarzenbach, G., Senn, H., and Andereg, G. (1957) Helv. Chim. Acta 40, 1886–1900
28. Deng, W. J., Robinson, J. M., Xing, J., and Cheung, H. C. (2003) J. Biol. Chem. 278, 32494–32499
29. Johnson, J. D., Nakamura, R. J., Vasulkas, C., and Smillie, L. B. (1994) J. Biol. Chem. 269, 8919–8923
30. El-sale, S. C., and Solaro, R. J. (1988) J. Biol. Chem. 263, 3274–3278
31. Gulati, J., and Babu, A. (1989) FEBS Lett. 245, 279–282
32. Morimoto, S., Ohta, M., Goto, T., and Ohtsuki, I. (2001) Biochem. Biophys. Res. Commun. 282, 611–615
33. White, S. P., Cohen, C. and Phillips, G. N., Jr. (1987) Nature 325, 826–828
34. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) Nature 424, 35–41
35. Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990) Nature 347, 37–44
36. Holmes, K. C., Popp, D., Gehbard, W., and Kabsch, W. (1990) Nature 347, 44–49
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