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# Conformational Changes of the Troponin–Tropomyosin Complex on F-Actin Observed by Fluorescence Resonance Energy Transfer Measurements

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Contraction of vertebrate striated muscle is regulated by the strong $Ca^{2+}$-dependent interaction among troponin (Tn), tropomyosin (Tm), and actin on the thin filament. Using fluorescence resonance energy transfer (FRET), the interactions between Tm and the Tn complex or between Tm and the Tn subunit, TnI or TnC, with or without other troponin subunits, were characterized in the presence or absence of F-actin and $Ca^{2+}$ ions. Cys-190 of Tm was selectively labeled with the acceptor probe, 4-dimethylaminophenylazophenyl 4′-maleimide. Troponin was selectively labeled at position 9 or 133 of TnI and position 98 of TnC with a donor probe, 5-(2-iodoacetylaminoethyl)aminonaphthalene 1-sulfonic acid. FRET measurements indicate that the interaction between TnI and Tm alone is very weak, but that in the presence of F-actin, TnI binds to the proper binding site on Tm even in the absence of TnT. The distances between Cys-190 of Tm on F-actin and Cys-9 or Cys-133 of TnI or Cys-98 of TnC in the reconstituted Tn were determined to be 52.8, 53.7, Å and 56.5 Å, respectively, in the absence of $Ca^{2+}$, indicating that the TnI–TnC complex, the globular portion of Tn, is located near Cys-190 of Tm on the reconstituted thin filaments. Upon binding of $Ca^{2+}$ to TnC, these distances increased by 5.6 and 1.4 Å or decreased by 5.4 Å, respectively. These $Ca^{2+}$-induced changes in Tn–Tm seem to occur only when F-actin is present, suggesting that the stable complex formation of TnI with the outer domain of F-actin upon removal of $Ca^{2+}$ is a very important event during inhibition.

**KEY WORDS:** Troponin; tropomyosin; skeletal muscle thin filament; $Ca^{2+}$ regulation; FRET.

## INTRODUCTION

$Ca^{2+}$ ions directly affect regulation of the myosin–actin interaction in vertebrate striated muscle through the action of tropomyosin (Tm)[5] and troponin (Tn) on muscle thin filaments [1]. Tn is a complex of TnC, TnI, and TnT. TnC binds $Ca^{2+}$, TnT attaches the Tn complex to Tm, and TnI is the inhibitory subunit which binds to the actin–Tm and inhibits actin–myosin interaction in the absence of $Ca^{2+}$. The key event in the regulation of muscle contraction is the $Ca^{2+}$-dependent change in the interactions between TnC and TnI. Numerous studies have characterized the interaction between the thin filament proteins to deduce how the $Ca^{2+}$-triggering signal is propagated from TnC to the rest of the thin filament (for reviews, see Refs.

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[5] *Abbreviations used*: DABMI, 4-dimethylaminophenylazophenyl 4′-maleimide; DTT, dithiothreitol; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid; FRET, fluorescence resonance energy transfer; IAEDANS, 5-(2-iodoacetylaminoethyl)aminonaphthalene 1-sulfonic acid; Tm, tropomyosin; Tn, troponin.



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2 and 3). It was demonstrated that the N-terminal domain of TnI interacts strongly with the C-terminal domain of TnC, irrespective of the calcium concentration (in the presence of magnesium), while the C-terminal plus inhibitory domain of TnI and the N-terminal domain of TnC interacts in a calcium-dependent manner [4]. The TnI–TnC complex binds to the C-terminal region of TnT to form a globular portion of Tn. TnT is an elongated protein [5] which lies extended along the C-terminal third of the Tm coiled coil from Cys-190 to the C terminus of Tm, where it overlaps the $NH_2$ terminus of the adjacent Tm [2]. The globular portion of Tn, the TnI–TnC complex, binds to Tm near Cys-190 of Tm [6].

Fluorescence resonance energy transfer (FRET) has been used extensively for studying the spatial relationships between residues on muscle proteins (see reviews in Refs. 7 and 8). This method is especially valuable for detecting a small conformational change, since the transfer efficiency is a function of the inverse in the sixth power of the distance between probes. Using this method, several attempts have been made to detect $Ca^{2+}$-dependent conformational changes of thin filaments to understand the regulation mechanism of Tn and Tm. Recently we used this method to test the notion of $Ca^{2+}$-dependent Tm movement on actin filaments as predicted by the steric blocking theory. FRET measurements between probes attached to Tm and actin did not detect any significant movement of Tm relative to actin on the reconstituted thin filament [9]. On the other hand, FRET measurements between probes attached to Cys-133 of TnI in the Tn complex and Lys-61 [10] or Cys-374 [11] of actin showed that the distance significantly decreased upon the removal of $Ca^{2+}$ from Tn. More detailed experiments suggest that during inhibition, the C-terminal region of TnI moves toward the outer domain of actin [12]. FRET measurements also indicated that the extent of this movement correlates well with the pCa dependence of tension development and that the time scale of this movement is rapid enough to allow this movement to be directly involved in regulation of muscle contraction [13].

In the present study, FRET between probes attached to Tn and Tm was measured in the presence and absence of $Ca^{2+}$ to understand the function of Tn as a molecular switch. A mutant TnI was expressed in which the natural cysteines (48, 64, and 133) were replaced with Ala or Ser and a single Cys residue was introduced at position 9 on the N-terminal region [14]. Cys-133 on TnI is located on the C-terminal region and is the most reactive cysteine in the Tn complex. Cys-98 on TnC is a natural single cysteine and is located on the helix region close to the C-terminal domain. Thus, Cys-133 or Cys-9 of TnI and Cys-98 of TnC were selectively labeled with a fluorescence resonance donor molecule, IAEDANS. On the other hand, Tm exists as a dimer of two polypeptide subunits, which associate in register in a coiled-coil fashion. The α subunit of rabbit skeletal muscle Tm has a single Cys residue at position 190 that is thought to be located near the binding site for the globular portion of Tn [6]. Cys-190 can be modified without greatly altering the biological activity of Tm, depending on the reagent. Thus, Cys-190 was labeled with the fluorescence resonance energy acceptor molecule, 4-dimethylaminophenylazophenyl 4′-maleimide (DABMI). FRET between these sites has been carried out to study the $Ca^{2+}$-dependent interaction between Tm and Tn or its subunits on F-actin filaments.

## MATERIALS AND METHODS

*Reagents.* Phalloidin from *Amanita phalloides* was purchased from Boehringer Mannheim Biochemica. IAEDANS and DABMI were from Molecular Probes. BCA protein assay reagent was from Pierce Chemicals. All other chemicals were analytical grade.

*Protein Preparations.* Actin and Tn from rabbit skeletal muscle were prepared as described in a previous report [10]. Cardiac Tm was prepared from rabbit hearts as reported previously [10]. Rabbit cardiac Tm has the same amino acid sequence as rabbit skeletal α-Tm peptide, which contains a single cysteine at position 190 [15]. Tn subunits were prepared according to the method of Ojima and Nishita [16] using CM-Toyopearl. Mutant TnI (Ala-9-Cys, Cys-48-Ala, Cys-64-Ala, Cys-133-Ser, and Trp-161-Phe) was expressed and purified as reported previously [14]. Protein concentrations were determined from absorbance $A_{290\,nm} = 0.63\,(mg/ml)^{-1}\,cm^{-1}$ for actin, $A_{280\,nm} = 0.33\,(mg/mL)^{-1}\,cm^{-1}$ for Tm, 0.45 for Tn, 0.18 for TnC, 0.372 for TnI, 0.122 for mutant TnI, and 0.458 for TnT. Concentrations of labeled proteins were measured with the Pierce BCA protein assay reagent. Relative molecular masses of 42,000 for actin, 66,000 for Tm, 18,000 for TnC, 21,000 for TnI, and 31,000 for TnT were used.

*Labeling of Proteins.* TnI was specifically labeled with IAEDANS in the Tn complex at Cys-133 as reported previously [10]. The labeled Tn complex was dissociated in 6 *M* urea and then separated into subunits using CM-Toyopearl according to the method of Ojima and Nishita [16]. Labeling of Cys-9 in a mutant TnI and Cys-98 of TnC with IAEDANS was carried out as reported previously [12]. Labeling of Tm at Cys-190 with DABMI was carried out as reported previously [10]. The absorption coefficients of 24,800 $M^{-1}\,cm^{-1}$ at 460 nm for

DABMI [17] and 6100 $M^{-1}$ cm$^{-1}$ at 337 nm for IAEDANS [18] were used for the determination of the labeling ratios. The typical labeling ratios were 0.48 for TnI, 0.43 for TnC, and 1.42 for Tm.

Reconstitution of the ternary Tn complex was carried out according to Farah *et al.* [4] as reported previously [12].

*Spectroscopic Measurements.* Absorption was measured with an Hitachi U2000 spectrophotometer. Steady-state fluorescence was measured with an Hitachi 850 fluorometer. Sample cells were placed in a thermostated cell holder.

*Fluorescence Resonance Energy Transfer.* The efficiency, $E$, of resonance energy transfer between probes was determined by measuring the fluorescence intensity of the donor in both the presence ($F_{DA}$) and the absence ($F_{D0}$) of the acceptor as given by

$$E = 1 - (F_{DA}/F_{D0}) \tag{1}$$

From the absorptions of the samples at the excitation ($A_{ax}$) and emission ($A_{em}$) wavelengths, the decrease in the fluorescence intensity due to inner filter effects was corrected, using Eq. (2), as reported previously [9, 10, 12].

$$F_{corr} = F_{obs} \times 10^{(A_{ex}+A_{em})/2} \tag{2}$$

According to Förster theory (see Ref. 19), the efficiency is related to the distance ($R$) between probes and Förster's critical distance ($R_0$) at which the transfer efficiency is equal to 50% by

$$E = R_0^6/(R_0^6 + R^6) \tag{3}$$

$R_0$ can be obtained (Å) by

$$R_0^6 = (8.79 \times 10^{-5}) n^{-4} \kappa^2 Q_0 J \tag{4}$$

where $n$ is the refractive index of the medium, taken to be 1.4, $\kappa^2$ is the orientation factor, $Q_0$ is the quantum yield of the donor in the absence of acceptor, and $J$ is the spectral overlap integral ($M^{-1}$ cm$^{-1}$ nm$^4$) between the donor emission $F_D$ ($\lambda$) and the acceptor absorption $\epsilon_A(\lambda)$ spectra defined by

$$J = \int F_D(\lambda)\, \epsilon_A(\lambda) \lambda^4 d\lambda / \int F_D(\lambda)\, d\lambda \tag{5}$$

The quantum yield was determined by comparing the integrated corrected fluorescence spectrum with that of quinine sulfate in 0.1 $N$ $H_2SO_4$, whose quantum yield was taken to be 0.70 [20]. $\kappa^2$ was taken as 2/3 for calculation of distances [8].

*Other Methods.* SDS-PAGE (3% stacking gel and 7–18% gradient separation gel) was carried out according to Laemmli [21]. ATPase activity was measured by the method of Tausky and Shorr [22]. The biological activity of the labeled Tm and Tn was assayed by determining the $Ca^{2+}$-dependent regulation of actoS1 ATPase in a fully reconstituted system as reported previously [9,12].

## RESULTS

In this work, the AEDANS moiety bound to Cys-9 or Cys-133 of TnI and Cys-98 of TnC was used as the energy-transfer donor, while DABMI bound to Cys-190 of Tm was used as the energy-transfer acceptor. The absorption spectrum of DABMI overlaps well with the fluorescence emission spectrum of AEDANS bound to Tn subunits. Since Tm is a dimer of $\alpha$-chains which contain a single cysteine residue at position 190, the energy acceptor molecule, DABMI, binds to Tm at a ratio of more than 1 (mol/mol). The analysis of fluorescence resonance energy transfer between a single donor and multiacceptors was described previously [23]. The fluorescence intensity $f$ in the presence of two acceptors is given by Eq. (6).

$$f = f_0/[1 + (R_0/R_1)^6 + (R_0/R_2)^6] \tag{6}$$

where $f_0$ is the fluorescence intensity in the absence of acceptor; and $R_1$ and $R_2$ are the distances between the donor and an acceptor attached to an $\alpha$-chain or another $\alpha$-chain of the same Tm molecule. Here we assume that fluorophores attached to Cys-190 on Tm are very close to each other and then the distances between the donor and the acceptor attached to one $\alpha$-chain or another $\alpha$-chain of Tm are the same. When both Cys-190s are fully labeled with acceptors, Eq. (6) becomes as follows.

$$f = f_0/[1 + 2(R_0/R)^6] = f_0/\{1 + [2^{1/6}(R_0/R)]^6\} \tag{7}$$

In this case, the apparent Förster's critical distance is $2^{1/6} \times R_0$ ($= 1.12R_0$). In the present study, the molar ratio of DABMI to Tm was 1.42. Therefore the apparent Förster's critical distance is $1.42^{1/6} \times R_0$ ($= 1.06R_0$), assuming random labeling of Cys-190 on Tm with DABMI.

The Tn complexes composed of these AEDANS-labeled Tn subunits retain their essential properties, being able to participate in the calcium regulation process as reported previously [12].

*FRET Between Cys-133 of TnI and Cys-190 of Tm.* The overlap integral $J$ was calculated to be $6.74 \times 10^{14}$ ($M^{-1}$ cm$^{-1}$ nm$^4$). By taking $n = 1.4$, $\kappa^2 = 2/3$, and $Q_0 = 0.31$, the Förster's critical distance $R_0$ and the apparent one were calculated to be 38.4 and 40.7 Å, respectively. The fluorescence spectra of AEDANS bound to Cys-133 of TnI on the Tn–Tm complex in

the presence and absence of acceptor (DABMI) were measured in 20 m*M* Tris–HCl (pH 7.6), 0.1 *M* KCl, 0.1 m*M* ATP, 1 m*M* EGTA (buffer $F_{-Ca}$). The molar ratio of Tm/Tn was 1:1. The excitation wavelength was 340 nm.

Figure 1 shows that the donor fluorescence in the absence of acceptor (curve 1) was strongly quenched in the presence of acceptor (DABMI) due to energy transfer (curve 2), indicating that TnI in the Tn complex is located close to Cys-190 of Tm. The addition of F-actin to the solution decreased the extent of quenching [1 − (curve 4/ curve 3)]. To obtain more quantitative data for the transfer efficiency, the ratio of donor fluorescence quenching was measured as follows. Two equivalent AEDANS–Tn (AEDANS bound to Cys-133 of TnI) solutions (2.2 ml of 0.02 mg/ml in buffer $F_{-Ca}$) in two sample cuvettes were prepared and their fluorescence intensities were measured at 490 nm. Then the same amounts of DABMI–Tm or non-labeled Tm were added to the cuvettes, and the ratio of those fluorescence intensities (in the presence and absence of acceptor) was taken. The apparent decrease in fluorescence intensity due to inner filter effects by the absorption of DABMI was corrected according to Eq. (2). Then the same amount of F-actin was added to the sample solutions and the ratio of those fluorescence intensities was taken. The ratio of the fluorescence intensities after the addition of 1.1 m*M* $CaCl_2$ was also measured. The efficiencies and calculated distances are summarized in Table I.

To understand the interaction between Tn subunits and Tm, the effects of F-actin, TnC, TnT, and $Ca^{2+}$ on

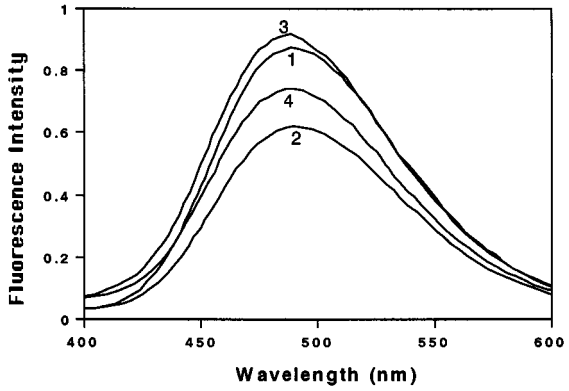


**Fig. 1.** Fluorescence spectra of AEDANS bound to Cys-133 of TnI on a reconstituted thin filament in the presence and absence of acceptor (DABMI). (1) AEDANS–Tn/Tm−Ca, (2) AEDANS–Tn/DABMI–Tm/−Ca, (3) AEDANS–Tn/Tm/F-actin/−Ca, (4) AEDANS–Tn/DABMI–Tm/F-actin/−Ca. Spectra were measured at 20°C in 0.1 *M* KCl, 2 m*M* $MgCl_2$,20 m*M* Tris–HCl (pH 7.6), 1 m*M* $NaN_3$, 0.1 m*M* ATP, and 1 m*M* EGTA (−Ca state). Concentrations of actin, Tm, and Tn were 0.26, 0.053, and 0.05 mg/ml, respectively. Excitation was at 340 nm.

**Table I.** (a) FRET Between Tn (Cys-133 of TnI) and Tm (Cys-190) and (b) FRET Between TnI (Cys-133) and Tm (Cys-190)[a]

a

| Added sample | Transfer efficiency $[1-(F/F_0)]$ | Distance *R* (Å) |
|---|---|---|
| Tn (donor)/−Ca | 0 | |
| Tm (acceptor) | 0.27±0.02 | 48.0±0.7 |
| FA | 0.16±0.02 | 53.7±1.4 |
| +Ca | 0.14±0.02 | 55.1±1.4 |

b

| Added sample | Transfer efficiency $[1-(F/F_0)]$ |
|---|---|
| TnI (donor)/−Ca | 0 |
| Tm (acceptor) | 0.04 |
| FA | 0.12 |
| TnC | 0.06 |
| TnT | 0.12 |
| +Ca | 0.06 |
| TnI (donor)/−Ca | 0 |
| TnT | 0 |
| Tm (acceptor) | 0.12 |
| FA | 0.10 |
| TnC | 0.14 |
| +Ca | 0.07 |

[a] The experimental errors in the transfer efficiencies were ±0.03.

FRET between TnI and Tm were studied. Two equivalent AEDANS–TnI solutions (2.2 ml of 0.02 mg/ml in buffer $F_{-Ca}$) in two sample cuvettes were prepared and their fluorescence intensities were measured at 490 nm as described for AEDANS–Tn. Then the same amounts of DABMl–Tm or nonlabeled Tm were added to the sample solutions. In this case, the fluorescence ratio ($F_{DA}/F_{D0}$) decreased little (0.96), indicating that the interaction between Tm and TnI alone is very weak. Then the same amount of F-actin was added to the sample solutions. The fluorescence ratio decreased appreciably (0.88), suggesting that TnI binds to Tm at the region close to Cys-190 in the presence of F-actin. The addition of TnC increased the ratio up to 0.94, but further addition of TnT decreased the ratio to 0.88. The results are summarized in Table I.

*FRET Between Cys-9 of TnI and Cys-190 of Tm.* The fluorescence spectrum of AEDANS bound to Cys-9 of TnI was similar to that of AEDANS bound to Cys-133 of wild-type TnI. The overlap integral *J* was calculated to be $6.74 \times 10^{14}$ ($M^{-1}$ $cm^{-1}$ $nm^4$). By taking $n = 1.4$, $\kappa^2 = 2/3$, and $Q_0 = 0.37$, the Förster's critical distance $R_0$ and the apparent one were calculated to be 39.5 and 41.9 Å, respectively. The transfer efficiency

between AEDANS bound to Cys-9 of mutant TnI in the troponin complex and DABMI bound to Cys-190 of Tm was measured in buffer $F_{-Ca}$ as described for Cys-133 of TnI in the troponin complex. The transfer efficiency was 0.14. The addition of F-actin increased the transfer efficiency up to 0.20, and further addition of 1.1 m*M* $CaCl_2$ decreased the transfer efficiency. The efficiencies and the calculated distances are summarized in Table II. The effects of F-actin, TnC, TnT, and $Ca^{2+}$ on FRET between TnI and Tm were also studied as described for the Cys-133-labeled TnI subunit. Similar results were obtained. The results are summarized in Table II.

*FRET Between Cys-98 of TnC and Cys-190 of Tm.* The quantum yield of AEDANS bound to Cys-98 of TnC in the Tn complex was 0.25 in the +Ca state and 0.23 in the −Ca state as reported previously [12]. The overlap integral $J$ was calculated to be $7.28 \times 10^{14}$ ($M^{-1}$ $cm^{-1}$ $nm^4$). $R_0$ and the apparent distance were calculated to be 37.5/37.0 and 39.7/39.2 Å (+Ca/−Ca), respectively. The transfer efficiency between AEDANS bound to Cys-98 of TnC in the Tn complex and DABMI bound to Cys-190 of Tm was measured in 20 m*M* Tris–HCl (pH 7.6), 0.1 *M* KCl, 0.1 m*M* ATP, and 50 μ*M* $CaCl_2$ (buffer $F_{+Ca}$) as described for TnI. The donor fluorescence was significantly quenched in the presence of acceptor due to energy transfer, indicating that, like TnI, TnC in the Tn complex is located close to Cys-190 of Tm. The addition of F-actin decreased the extent of quenching slightly, and the removal of $Ca^{2+}$ by the addition of EGTA increased the extent of quenching. The efficiencies and the calculated distances are summarized in Table III. The effects of F-actin, TnI, TnT, and $Ca^{2+}$ on FRET between the TnC subunit and Tm were also studied as described for TnI. The results are summarized in Table III.

The ratio of donor fluorescence quenching was measured by titrating AEDANS–Tn (Cys-98 of TnC) with DABMI–Tm (Cys-190) in the +Ca (buffer $F_{+Ca}$) or −Ca (buffer $F_{-Ca}$) state and in the presence or absence of F-actin (Fig. 2). The fluorescence intensity was measured at 490 nm. For correction of the fluorescence intensity change of AEDANS–Tn upon binding to Tm, the same amount of nonlabeled Tm was added to the AEDANS–Tn solution, and the ratio of these fluorescence intensities

**Table II.** (a) FRET Between Tn (Cys-9 of mTnI) and Tm (Cys-190) and (b) FRET Between mTnI (Cys-9) and Tm (Cys-190)[a]

a

| Added Sample | Transfer efficiency $[1-(F/F_0)]$ | Distance $R$ (Å) |
|---|---|---|
| Tn (donor)/−Ca | 0 | |
| Tm (acceptor) | 0.14 ± 0.02 | 56.7 ± 1.7 |
| FA | 0.20 ± 0.02 | 52.8 ± 1.1 |
| +Ca | 0.12 ± 0.02 | 58.4 ± 2.0 |

b

| Added sample | Transfer efficiency $[1-(F/F_0)]$ |
|---|---|
| mTnI (donor)/−Ca | 0 |
| Tm (acceptor) | 0.05 |
| FA | 0.18 |
| TnC | 0.10 |
| TnT | 0.14 |
| +Ca | 0.11 |
| mTnI (donor)/−Ca | 0 |
| TnT | 0 |
| Tm (acceptor) | 0.22 |
| FA | 0.22 |
| TnC | 0.15 |
| +Ca | 0.11 |

[a] The experimental errors in the transfer efficiencies were ±0.03.

**Table III.** (a) FRET Between Tn (Cys-98 of TnC) and Tm (Cys-190) and (b) FRET Between TnC (Cys-98) and Tm (Cys-190)[a]

a

| Added sample | Transfer efficiency $[1-(F/F_0)]$ | Distance $R$ (Å) |
|---|---|---|
| Tn (donor)/+Ca | 0 | |
| Tm (acceptor) | 0.22±0.02 | 49.0±1.0 |
| FA | 0.18±0.02 | 51.1±1.2 |
| −Ca | 0.10±0.02 | 56.5±1.1 |

b

| Added sample | Transfer efficiency $[1-(F/F_0)]$ |
|---|---|
| TnC (donor)/+Ca | 0 |
| TnI | 0 |
| Tm (acceptor) | 0.02 |
| FA | 0.02 |
| −Ca | 0.04 |
| TnC (donor)/+Ca | 0 |
| TnI | 0 |
| TnT | 0 |
| Tm (acceptor) | 0.20 |
| FA | 0.16 |
| −Ca | 0.17 |
| TnC (donor)/+Ca | 0 |
| TnT | 0 |
| Tm (acceptor) | 0.23 |
| FA | 0.12 |
| TnI | 0.12 |
| −Ca | 0.11 |

[a] The experimental errors in the transfer efficiencies were ±0.03.

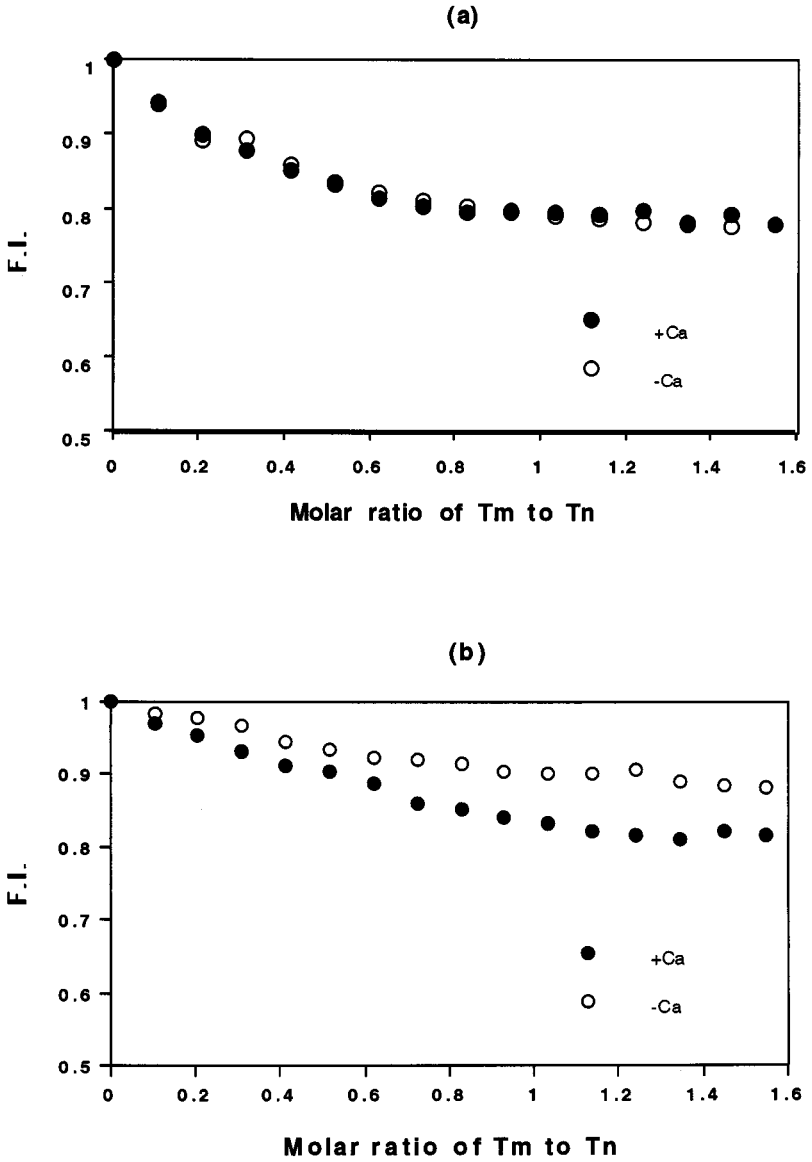


**Fig. 2.** (a) Relative fluorescence intensity of AEDANS bound to Cys-98 of TnC in the Tn complex vs. molar ratio of DABMI–Tm to Tn. Values were obtained at 20°C in 30 m*M* KCl, 2 m*M* $MgCl_2$, 20 m*M* Tris–HCl (pH 7.6), 1 m*M* $NaN_3$, 0.1 m*M* ATP, and 50 $\mu M$ $CaCl_2$ (+Ca state; fill circles) or 2 m*M* EGTA (−Ca state; open circles) after correction of the inner filter effects according to Eq. (2). The concentration of AEDANS–Tn was 0.05 mg/ml. Excitation was at 340 nm and emission was measured at 490 nm. (b) Relative fluorescence intensity of AEDANS bound to Cys-98 of TnC on the Tn complex in the presence of F-actin vs. molar ratio of DABMI–Tm to Tn. Values were obtained at 20°C in 30 m*M* KCl, 2 m*M* $MgCl_2$, 20 m*M* Tris–HCl (pH 7.6), 1 m*M* $NAN_3$, 0.1 m*M* ATP, and 50 $\mu M$ $CaCl_2$ (+Ca state; fill circles) or 2 m*M* EGTA (−Ca state; open circles) after correction of the inner filter effects according to Eq. (2). Concentrations of AEDANS–Tn and F-actin were 0.05 and 0.2 mg/ml, respectively. Excitation was at 340 nm and emission was measured at 490 nm.

was taken. The apparent decrease in fluorescence intensity due to inner filter effects by the absorbance of DABMI–Tm was corrected according to Eq. (2). The fluorescence ratio decreased in the range of the Tm/Tn molar ratio up to 1 and became almost-constant in the range over 1. In the absence of F-actin, no difference was observed between the +Ca and the −Ca states. However, in the presence of F-actin, the extent of fluorescence

quenching was greater in the +Ca state than in the −Ca state. From the saturated points, the transfer efficiencies were taken to be 0.22 in the absence of F-actin, 0.18 in the presence of F-actin and the +Ca state, and 0.10 in the presence of F-actin and the −Ca state.

## DISCUSSION

In the calculation of the distances between probes, the value of 2/3 was used for the orientation factor $\kappa^2$. In the present study, we used AEDANS bound to Cys-9 or Cys-133 of TnI and Cys-98 of TnC as the donor molecule. The limiting anisotropy was measured to be 0.331, 0.205 and 0.179, respectively [12]. These values are significantly lower than the theoretical limit of 0.4, indicating that the donor attached to these sites is substantially depolarized by either rapid segmental motion or electronic mechanism. It has been argued by several authors that the choice of 2/3 for $\kappa^2$ is not an unreasonable one due to electronic and rotational depolarization mechanisms [8, 24]. We have pointed out that reasonable agreement was obtained between intra- and the intermolecular distances in G-actin and F-actin determined by FRET by assuming $\kappa^2 = 2/3$ and the distances determined by X-ray diffraction data [7]. Therefore, our use of $\kappa^2 = 2/3$ appears to be empirically justified.

The transfer efficiency is a function of the inverse of the sixth power of the distance between probes. It is 0.1 for $R = 1.44\ R_0$ and only 0.05 for $R = 1.63\ R_0$. In the present study $R_0$ is about 41 Å. Thus intermolecular energy transfer occurs only when two labeled proteins interact closely. FRET is very useful for studying the interaction between Tn subunits and Tm and their $Ca^{2+}$-dependent changes under physiological conditions. Tables I, II, and III show qualitative aspects about the interactions of TnI or TnC with other Tn subunits, Tm, and F-actin. In the case of TnI and Tm (Tables I and II), the transfer efficiencies were very low in the absence of F-actin, but in the presence of F-actin, the efficiencies were very close to the values for whole troponin. The results indicate that the interaction between TnI and Tm alone is very weak but that, in the presence of F-actin, TnI binds to the proper binding site on Tm (near Cys-190) even in the absence of TnT. However, the addition of TnC weakens the interaction between TnI and Tm-F-actin even in the absence of $Ca^{2+}$, but the further addition of TnT recovers the interaction. On the other hand, in the case of TnC and Tm (Table III), the transfer efficiency was trivial even in the presence of Tm-F-actin and TnI. The presence of TnT is essential for TnC to interact with Tm. It is well known that inhibition of the actin–myosin interaction by TnI is potentiated by Tm, partially relieved by TnC (−Ca state) and fully released by TnC in the presence of $Ca^{2+}$. This is consistent with the present FRET measurements. Thus the interaction between Tn subunits and Tm and their $Ca^{2+}$-dependent changes can be easily studied by measuring the transfer efficiency.

Flicker *et al.* [6] showed that TnC, TnI, and part of TnT bind near Cys-190 of Tm, about one-third of the way along the molecule, with the rest of TnT extending toward the COOH terminus. Tao *et al.* [25] demonstrated that αα-Tm specifically labeled at Cys-190 with photo-cross-linker can be cross-linked with TnT, TnI, and TnC. These results are in accordance with the present FRET results, showing that TnC and TnI bind Tm near Cys-190. However, Tao *et al.* [25] did not detect any $Ca^{2+}$-dependent change in the cross-linking. Sutoh [26] studied the formation of photo-cross-linking between TnC and other components in contact with it and detected an appreciable amount of cross-linking between TnC and TnI or TnT. No effect of calcium on the cross-linking in Tn alone was observed, but in the presence of F-actin–Tm, the extent of cross-linking was sensitive to calcium and magnesium ions. On the other hand, Luo *et al.* [27] reported that the distance between Cys-48 and Cys-133 of TnI in the ternary Tn complex increases upon removal of $Ca^{2+}$ in the reconstituted thin filament but not in the Tn–Tm complex without F-actin. These results are consistent with the results in Fig. 2. In the absence of F-actin, the distance between probes on Cys-190 of Tm and Cys-98 of TnC was not sensitive to calcium ions, but in the presence of F-actin, it increases when calcium ion are removed. During inhibition, the C-terminal and inhibitory regions of TnI decrease the affinity for the N-terminal domain of TnC [4] and moves toward the outer domain of actin [12]. Also, TnI and TnC change position on Tm. Consequently the distance between Cys-133 of TnI and Cys-190 of Tm decreased, while the distance between Cys-98 of TnC and Cys-190 of Tm increased. This series of conformational changes in Tn–Tm seems to occur only when F-actin is present, suggesting that a stable complex formation of TnI with the outer domain of F-actin upon removal of $Ca^{2+}$ is a very important event during inhibition.

It should be mentioned that the $Ca^{2+}$-induced change in distance between Cys-133 of TnI and Cys-190 of Tm (1.4 Å) is significantly smaller than those between Cys-133 of TnI and residues (Gln-41, Lys-61, Cys-374, the nucleotide binding site) on the outer domain of actin (more than 10 Å; see Ref. 12). On the other hand, the distance between Cys-190 of Tm and residues of the outer domain of actin did not change significantly [9]. In summarizing these results, Fig. 3 shows a model for

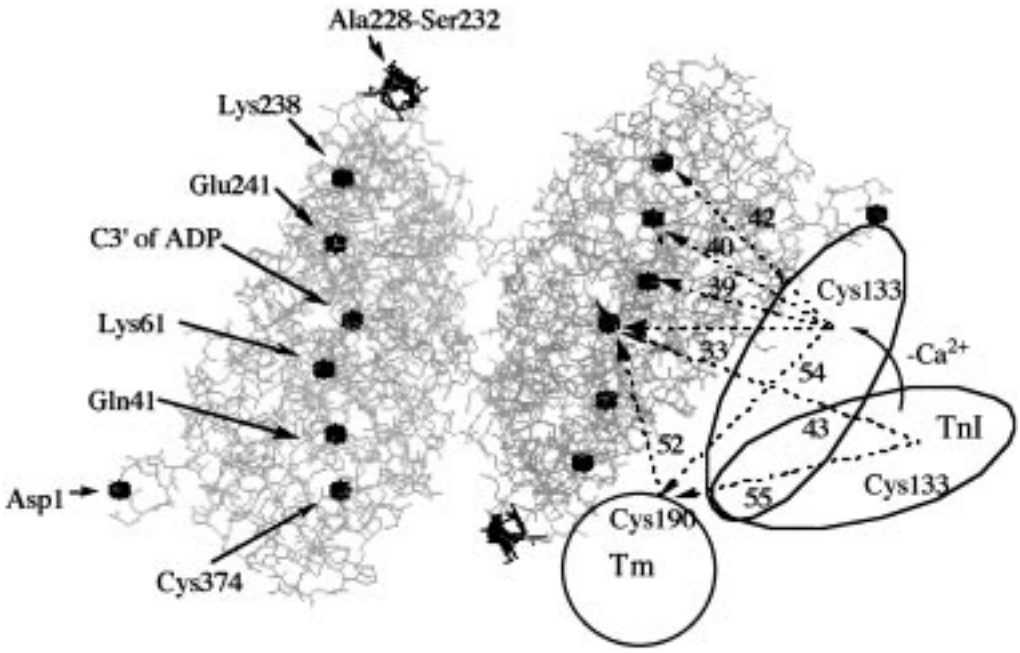


**Fig. 3.** A schematic model of the $Ca^{2+}$-induced movement of TnI relative to F-actin and Tm. The distances between Cys-133 of TnI and residues of actin or Cys-190 of Tm are indicated in the presence and absence of $Ca^{2+}$. F-actin is constructed of two monomers using the atomic coordinates of Lorenz *et al*. (28) and the cross-section view from the pointed end is shown. Cα of Asp-1, Gin-41, Lys-61, Lys-238, Glu-241, and Cys-374 and C3′ of bound ADP are shown by fill circles, and residues 228–232 are shown with the ribbon structure on the wireframe model of F-actin. The software used for the figure was Rasmol by Roger Sayle. The position of Tm (in the presence of $Ca^{2+}$) is from Saeki *et al.* (29).

the $Ca^{2+}$-induced movement of TnI relative to F-actin–Tm. This model is consistent with the previously proposed model for the regulation mechanism by tropomyosin and troponin, in which, during inhibition, TnI moves toward the outer domain of actin like a lever arm and consequently cross-links the outer domain of actin with tropomyosin, which covers seven actin inner domains along the long-pitch helix [9].

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