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Research paper

# TROPONIN REGULATORY INTERACTION ON THE THIN FILAMENT

**Ravi K Gawalapu* Patrick Studtman Emily Vargo Darl R Swartz**

Purdue University, Department of Animal Sciences, 901 State St., W. Lafayette, IN 47907-2054

*corresponding author: ravi_kumar.gawalapu@jsums.edu

**ABSTRACT: In order to understand the spatial rearrangements of thin filament proteins during the regulation of muscle contraction, we used fluorescence resonance energy transfer (FRET) to measure $Ca^{2+}$ dependent, Actin-Tm(Ac-Tm), myosin-induced distance changes between troponin I (TnI) and troponin C (TnC). We labeled the single Cys-134 of sTnI and residue 10 of sTnC with Alexa $C_5$ 488 and 594 maleimide respectively. These fluorescent probes were used as donor and acceptor respectively, for the FRET measurements. We reconstituted a troponin complex which contained the Alexa 488-labeled troponin I(TnI) ,troponin T (TnT), Alexa 594 labeled troponin C (TnC). The magnitude of distance changes at low $Ca^{2+}$ is higher than at high $Ca^{2+}$ both in presence of Ac-Tm and myosin (S1). The extent of energy transfer was determined by measuring the quenching of the donor fluorescence decay. Steady state fluorescence measurements showed that in the presence of the acceptor, donor fluorescence was quenched 50 – 85% and the quenching was sensitive to calcium, actin-Tm and rigor S1 in the presence of actin-Tm. The results indicate that the distance between these two sites is not fixed, suggesting that the protein regions involved possess considerable segmental flexibility.**

**KEYWORDS: FRET (Fluorescence resonance energy transfer), thick filaments, thin filaments, muscle contraction, myosin, actin, tropomyosin, troponin I, troponin C, troponin T, labeling.**

## INTRODUCTION

Vertebrate skeletal muscle contraction is the result of a cyclic interaction of thick myosin filaments with the thin filaments. Troponin (Tn) is the primary switch, which causes these two sets of filaments to slide past each other (Huxley et al.1954). Tn consists of three units (TnI, TnT, and TnC) and is located periodically on a thin filament with tropomyosin (Tm). 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 (Gordon et al.2000). Calcium binding to the thin filament protein troponin is required for cardiac and skeletal muscles to contract, and several studies indicate that this regulation involves shifting the tropomyosin position on the actin filament (Ebashi et al.1969).

The interaction of myosin with actin is driven by the hydrolysis of ATP by myosin in a reaction which is activated by actin and regulated by tropomyosin (Tm) and troponin (Tn) on actin filaments in response to a change in $Ca^{2+}$ concentration from approximately $10^{-7}$ to $10^{-5}$ M (Ebashi et al. 1969). The proteins troponin and tropomyosin are responsible for this effect of $Ca^{2+}$ on the interaction between myosin and actin (Perry et al.1979).

The Tn core domain has two characteristic subdomains: (1) the ñregulatory head,ò composed of the N-terminal region of TnC and the C-terminal region of TnI, and (2) the ñI-T arm,ò composed of long coiled-coil regions from TnT2, TnI and the C-terminal lobe of TnC. The relative orientation of the regulatory head and the I-T arm may play a fundamental role in the regulatory mechanism. Although 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, the mechanism of this regulatory process is still not well understood as many components of the regulatory machinery are missing from the crystal structure of the core complex, including parts of TnI and TnT, Tm and actin. The detailed positions of Tn subunits on the thin filament and their changes

in response to the on and off states of the thin filament are not yet known. Therefore, these are ideal sites for incorporation of FRET probes to monitor the molecular mechanisms involved in thin filament regulation.

Fluorescence resonance energy transfer (FRET) has been extensively used for studying the spatial relationships between residues on muscle proteins (Remedios et al.1987) and recently computational methods have been used to study conformational changes in myosinS1( Gawalapu et al.2006). FRET is especially valuable for detecting a small conformational change and distance changes in the range of 10-70 Å, since the transfer efficiency is a function of the inverse of the sixth power of the distance between probes. For this method, fluorescence donor and acceptor molecules are specifically labeled so that the assignment of the conformational change is direct. Furthermore, this method reveals the dynamic structures of a protein assembly in solution and can detect a very rapid conformational change, *i.e.* on a milliseconds time scale, in combination with a stopped flow apparatus (Miki et al.1993).

In the present study, we generated single cysteine mutants of rabbit skeletal TnC and TnI. TnC in the N helix (S10C) and TnI in the mobile domain (134C) were labeled with Alexa 594 maleimide (acceptor) and Alexa 488 maleimide (donor) respectively and reconstituted into the ternary Tn complex. Figure 1 shows a schematic representation of the labeled proteins. We measured intermolecular FRET between probes attached to Tn subunits on the reconstituted thin filament in the presence and absence of $Ca^{2+}$. Cys134 of TnI was chosen because the region containing this residue undergoes reversible $Ca^{2+}$ dependent movements between actin and TnC (Tao et al.1990) and has been suggested to play an important role in the regulation process (Kobayashi et al.1994). The extent of energy transfer was determined by measuring the quenching of the donor fluorescence decay.

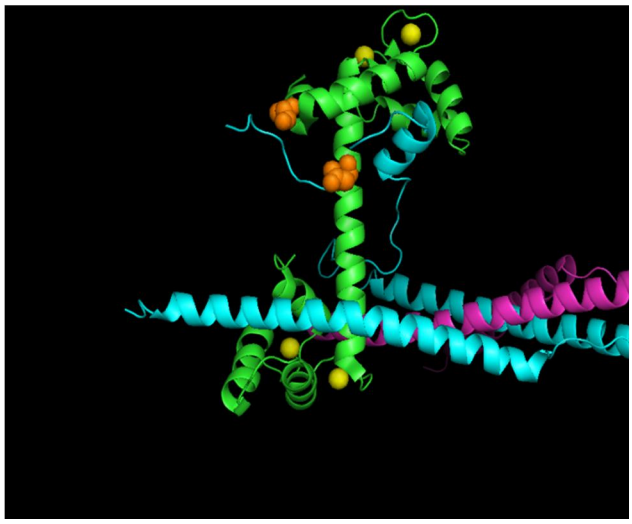

Figure1: Location of donor and acceptor sites in calcium saturated Tn. Troponin C is green, TnI is turquoise, and the labeling sites are shown as orange spheres. The donor site is in the mobile domain of TnI and the acceptor site is in the N-helix of TnC. The image was rendered using MacPyMOL and structure 1YTZ.

## MATERIALS & METHODS

### Protein isolation

Rabbit white muscle was used as the source for skeletal myofibrils and skeletal Tn. sTn, sTnT, were isolated by methods (Potter et al.1980) with minor modifications. Myofibrils were isolated from rabbit psoas as described in (Swartz et al.1990). sTn was labeled with Alexa488, Alexa594 maleimide (Invitrogen, Carlsbad, CA) as described in (Swartz et al.2006).

### Solutions

Buffers for Tn exchange experiments were made to specified free calcium levels (pCa 9.0 to 4.0) and contained 20 mM PIPES (pH 7.0), 4 mM EGTA, 4 mM (free) $Mg^{2+}$, 1 mM $NaN_3$, 1 mM DTT, 1 mg/mL BSA (unless noted otherwise), total calcium to get the specified free calcium, and 200 mM ionic strength via KCl. Buffers for ATPase assays contained 1 mM MgATP with total $Mg^{2+}$, calcium, and KCl adjusted to give the same free $Mg^{2+}$ and calcium levels and ionic strength as for the exchange experiments.

### Labeled Tn exchange reaction

Myofibrils in pCa 4.0 buffer were incubated at 25°C three times for 2 h each using 0.25 mg/mL Tn and 0.5 mg/mL myofibrils. The myofibrils were collected by centrifugation at 1000 × *g* for 10 min, and the supernatant removed and replaced with an equal volume of pCa 4.0 buffer containing 0.25 mg/mL Tn. After the third exchange, the myofibrils

were collected by centrifugation, washed twice with 0.33 volumes (relative to the initial volume) of pCa 9.0 buffer, and resuspended in a minimal volume of pCa 9.0 buffer, and the myofibril protein concentration was measured. Myofibrils were stored on wet ice and used within 4 days. For pCa 9.0 and 4.0 ATPase assays, exchange was done with labeled Tn.

**Assays**

Labeled protein concentration was determined using the BCA assay with unlabeled proteins as standards and extinction coefficients (at 280 nm) of 0.45 mL/mg for sTn. The molecular weight of the proteins used to calculate concentrations were 70,000 g/mol for sTn. Labeling ratios were 0.9ï 1.1 mol dye/mol protein. Myofibril protein concentration was determined by the Biuret assay with BSA as the standard. ATPase assays were done at room temperature as described in (Swartz et al.1997) in a final volume of 100 $\mu$L at 0.1 mg/mL myofibril protein using the buffers described above. Fig 2 shows the ATPase activity of the mutant proteins. The data were fitted to a sigmoid logistic equation to obtain the $pCa_{50}$ and $n_H$ with GraphPad Prism software (GraphPad Software, La Jolla, CA). Statistical analysis was done using SAS 9.2(SAS Institute Inc, Cary, NC).

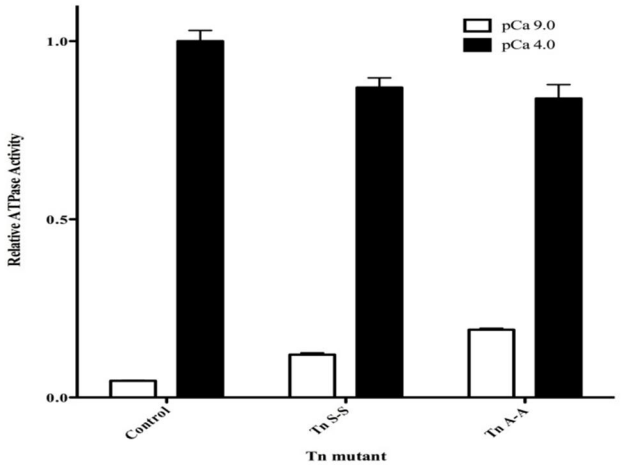


Figure 2: Myofibrillar ATPase activity of $Tn_{da}$ mutants exchanged into myofibrils.

**Proteins**

Single cysteine mutants of rabbit TnC (S10C) and TnI (134C) were made by site-directed mutagenesis and confirmed by sequencing. Troponin T and single cysteine mutants were expressed in E. coli and purified by ion exchange, phenyl-sepharose (S10C sTnC) and TnC-sepharose (sTnI 134C) chromatography. Labeling of reduced and denatured TnI and TnC were done using

Alexa$C_5$488 and 594 maleimide respectively. The Tn complex was reconstituted in 6M urea using 1 TnT:1.2 TnI:1.2 TnC, dialyzed against 200 mM KCl, 10 mM MOPS, 1 mM $MgCl_2$, 1 mM DTT, 0.45 micron filtered and the trimer isolated by Sephacryl S-200 chromatography. The TnIC donor/acceptor complex was made in a similar fashion using 1 mole TnI to 1 mole TnC. Tn trimer complexes included unlabeled TnT, donor only Tn ($Tn_d$, Alexa 488 TnI) and donor/acceptor Tn ($Tn_{da}$, Alexa 488 TnI and Alexa 594 TnC). Protein concentration was measured by the BCA assay using unlabeled Tn as the standard. The labeling ratio was determined by spectroscopy with the dye: protein being 0.9-1.1 for both proteins. The trimer fractions collected from Sephacryl S-200 chromatography were subjected to spectroscopy measurements at 280,496 and 590 nm. The fractions which contained similar ratios for 496:590,280:590 and 280:496 were pooled together and concentrated. Similarly, trimer fractions for samples labeled to TnI only were subjected to spectroscopy measurements at 280 and 496. The fractions which contained similar ratios for 280:496 were pooled together and concentrated. Actin, tropomyosin and S1 were purified by standard methods. Actin-Tm was made by mixing 7 mole actin:7mole phalloidin:1 mole Tm. TnT was exchanged into myofibrils and these myofibrils were used to monitor function of the labeled $TnIC_{da}$ complex.

**Fluorescence spectroscopy**

Fluorescence was measured in a Perkin Elmer LS50B spectrometer with excitation at 485 nm and emission scanning (510 ï 650) or a fixed wavelength of 518 nm. Band pass was 10 nm for excitation and emission monochrometors. Fig 3 shows the emission spectra of the protein. The sample (3 ml) was thermostated at 20°C and constantly stirred. Assay buffer was 200 mM MOPS (pH 7.2), 4 mM $MgCl_2$, 2 mM EGTA, 1mM DTT, 0.02% Tween 20 and K-propionate to an ionic strength of 200 mM. The efficiency (E) of resonance energy transfer between probes was determined by measuring the fluorescence intensity of the donor in both the presence ($Tn_{da}$) and absence ($Tn_d$) of the acceptor at 518 nm. Distances were estimated from the E assuming a $R_o$ of 60Å.

Calcium titrations were done by adding 10µl volumes of a stock calcium solution made in assay buffer and incubated for two minutes. Fluorescence intensity and free calcium concentrations were corrected for dilution.

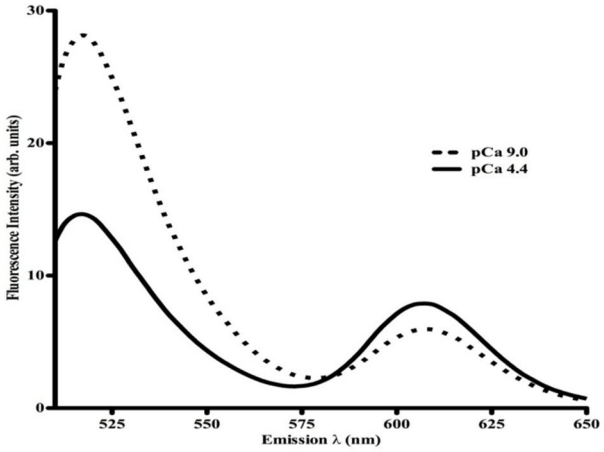


**Figure 3:** Emission spectra of Tnda S-S at high and low calcium. The donor alone peak intensity was 95 and 103 at pCa 9.0 and 4.4 respectively under the same conditions.

Troponin dissociation was obtained by adding 50 nM of labeled Tn to buffer and fluorescence intensity value is recorded until there was no change in fluorescence intensity, once the fluorescence reading is stable and constant. Then, 1 uM of unlabeled TnC is added and allowed to react until there was no change in fluorescence intensity and then saturated levels of Actin-Tm is added and allowed to react until there was no change in fluorescence intensity and then saturating amounts of rigor S1 is added and allowed to react until there was no change in fluorescence intensity. The reaction time for each addition varied with different mutant. Similarly, Troponin dissociation of troponin complex only was done by adding 10 nM of labeled Tn to buffer and allowed to react. The reaction was followed for 1000sec. Fig 4 shows the Troponin dissociation of the four mutants.

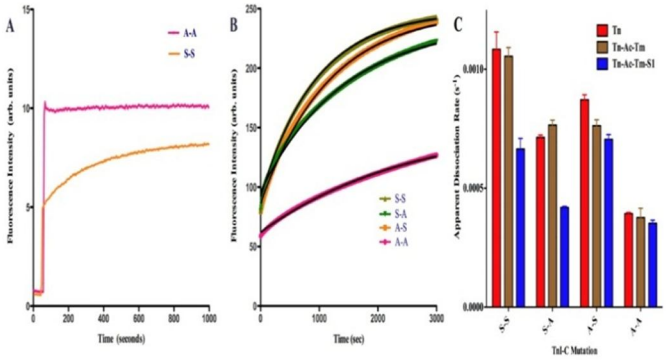


**Figure 4:** Troponin complex dissociation upon dilution and after addition of TnC at low calcium. Upon dilution of Tn (10 nM final) into pCa 9.0 buffer (50s), there was a gradual increase in intensity for the S-S mutant but essentially no change for the A-A mutant as shown in graph A.

Addition of unlabeled TnC (1 uM) to the Tn mutants (50 nM) at pCa 9.0 resulted in slow increase in fluorescence with the rate being dependent on the mutant as shown in B. The rates were fitted (solid black line in B) to estimate the dissociation rate. The apparent dissociation rate was also dependent on actin-Tm (Tn-Ac-Tm) and S1 (Tn-Ac-Tm-S1) and mutant Tn with the S-S mutant being most sensitive and the A-A mutant not being sensitive as shown in graph C. A-A mutant is significantly different from the other three proteins.There is no difference between the other three proteins when Ac-Tm is added. Tn complex having A-A substitution for unlabelled cysteines undergoes large fluorescence changes when excess TnC, Ac-Tm, S1 is added.

## RESULTS AND DISCUSSION

To study the proximity between specific residues of Tn complex in the reconstituted thin filament and the changes in response to a change in $Ca^{2+}$ concentration, FRET efficiencies between probes attached to the TnI and TnC subunits were measured in the presence and absence of $Ca^{2+}$. The energy donor molecule Alexa-488 is attached to cysteine residues at position 134 of TnI and energy acceptor molecule Alexa-594 is attached to cysteine residues at position 10 of TnC. The cysteines in TnI and TnC except the labeled residue(134 for TnI and 10 for TnC) were replaced only with alanine, serine and also a combination of alanine and serine and FRET was performed to see the distance changes( Table 1). The present results showed that not only the regulatory region but also whole TnI moves on the thin filament in response to the three states of the thin filament. The present FRET measurements provide structural evidence for three states of thin filaments(relaxed, $Ca^{2+}$-induced or closed, and Sl-induced or open State) for the regulation mechanism of skeletal muscle contraction.

| Tn Mutant | pCa 9.0 | | | pCa 4.4 | | |
|---|---|---|---|---|---|---|
| | Tn | Tn-Ac-Tm | Tn-Ac-Tm-S1 | Tn | Tn-Ac-Tm | Tn-Ac-Tm-S1 |
| S-S | 51.2(.098) | 56.5(.18) | 52.2(.11) | 43.8(.079) | 45.7(.170) | 44.3(.142) |
| S-A | 50.4(.163) | 55.3(.43) | 50.2(.020) | 43.5(.055) | 47.9(.413) | 44.1(.36) |
| A-S | 50.2(.425) | 56.6(.344) | 51.0(.069) | 44.4(.245) | 48.3(.280) | 44.8(.454) |
| A-A | 50.9(.216) | 54.0(.141) | 51.4(.141) | 46.9(.026) | 48.0(.124) | 47.1(.298) |

**Table 1:** Calculated mean distances between the mobile domain of TnI and N-helix of TnC for the different Tn mutants as Tn alone, and on the thin filament with and without S1 at high and low calcium. Transfer efficiency was calculated assuming a $R_o$ of 60Å. Data represent the mean of 3 observations. Table 2 shows that $Ca^{2+}$ sensitivity was not significantly changed by the presence of actin-Tm alone and with S1. Calcium titrations of the fluorescence change associated with the distance change shows that $Ca^{2+}$ sensitivity is the lowest for actin-Tm and highest for actin-Tm-S1 with Tn alone being intermediate. Actin-Tm caused a decrease in $Ca^{2+}$ sensitivity consistent with it increasing the distance between the N-helix of TnC and the C-domain of TnI. As expected, rigor S1 caused an increase in calcium sensitivity consistent with it decreasing the distance between the donor and acceptor sites in the reguatory domains.

| Tn Mutant | $pCa_{50}$* | | | $n_H$* | | |
|---|---|---|---|---|---|---|
| | Tn | Tn-Ac-Tm | Tn-Ac-Tm-S1 | Tn | Tn-Ac-Tm | Tn-Ac-Tm-S1 |
| S-S | -6.77 (0.0025) | -6.64 (0.0049) | -6.89 (0.0039) | 1.13 (0.0080) | 0.934 (0.012) | 1.06 (0.011) |
| S-A | -6.33 (0.0057) | -6.25 (0.0064) | -6.51 (0.0098) | 1.14 (0.017) | 0.911 (0.013) | 0.745 (0.016) |
| A-S | -6.41 | -6.23 | -6.44 | 1.12 | 0.89 | 1.10 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (0.00 38) | (0.00 86) | (0.00 61) | (0.01 1) | (0.0 17) | (0.0 18) |
| A-A | -6.47 (0.00 96) | -6.27 (0.01 0) | -6.61 (0.00 66) | 1.17 (0.03 2) | 1.03 (0.0 26) | 1.14 (0.0 21) |

**Table 2:** Calcium titration of the different Tn mutants alone and on the thin filament without and with S1.

* Fitted value (± standard error of the fit), n=3.

**Conclusions independent of mutant**:

The greatest distance between the regulatory domains is at pCa 9.0 with actin-Tm having greater effect at low calcium than high calcium. The distance is larger than in Tn alone suggesting that the mobile domain binds to a site on actin-Tm and moves further away from the N-helix. Addition of calcium causes a large change in the distance between the mobile domain and N-helix suggesting that the mobile domain moves from its site on actin-Tm towards the N-helix. The distance changes between the mobile domain of TnI and the N-helix of TnC has the least distance change at pCa 9.0 with rigor S1. The data shows that rigor S1 binding affects the conformation of calcium saturated Tn and rigor S1 moves the regulatory domain of TnI closer to TnC which may explain the observed enhanced calcium sensitivity in the presence of S1. Rigor cross-bridges decreases the distance between the mobile domain and N-helix for Tn-actin-Tm at low calcium. This suggests that rigor cross-bridges displace the mobile domain from its actin-Tm site and move it closer to the N-helix. The calcium sensitivity of the probe bound to actin-Tm is increased by strong cross-bridges. This increased sensitivity is likely caused by partial movement of the mobile domain from actin-Tm towards the N-helix by the rigor cross-bridge. These two-step changes in distances provide a direct link of structural changes at the interface between sTnI and sTnC to the three-state model of thin filament regulation of muscle contraction and relaxation.

**Conservative mutation effects**

The choice of conservative cysteine mutations for the non-labeled cysteine in the TnI and TnC

subunits, in terms of either a hydrophilic serine or a hydrophobic alanine, influences the calculated distances. Interestingly, the choice of conservative mutations did not influence the distance measurement in Tn alone at pCa 9.0. The differences were most apparent in other Tn states (pCa and /or actin-Tm).In general, conservative serine mutations result in larger apparent distance changes than alanine mutations. The enhanced calcium sensitivity of alanine mutations may result from a closer or more rigid positioning of the regulatory domain of Tn closer to the regulatory domain of TnC. Hybrid complexes containing alanines in either TnC or TnI gave values more similar to all serine mutants at pCa 9 actin-Tm-S1 and pCa 4.4 actin-Tm. Hybrid complexes containing alanines in either TnC or TnI gave values more similar to the all serine mutants at pCa 9 actin-Tm-S1 and pCa 4.4 actin-Tm.

Upon binding of $Ca^{2+}$to TnC the distance between sTnI and sTnC decreases which corresponds to a $Ca^{2+}$induced conformational change but the presence of serine for unlabelled cysteines contributes significantly to the distance change which may be due to intramolecular dissociation. The FRET distance measurements showed that bound $Ca^{2+}$ induced large increases in the distances between sTnI and sTnC on thin filaments. The distance is lowest when ala I-ala C are substituted for unlabelled cysteines but the distance increases as serine is introduced, the maximum distance change is 11 Å when serine is used to substitute unlabelled cysteines on both TnI and TnC. This explains that the serines are causing intramolecular dissociation which corresponds to a $Ca^{2+}$induced conformational change of thin filaments. Strongly bound myosin S1 induced additional increases in these distances in the presence of bound $Ca^{2+}$.

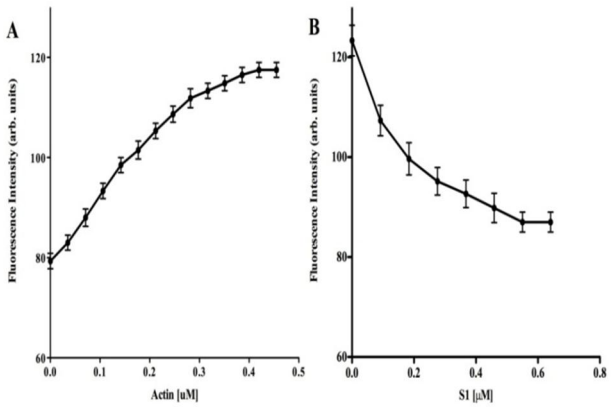


**Figure 5:** Titration of Tn with actin-Tm and S1 titration of Tn-actin-Tm with S1 at low calcium. Actin-Tm increases the distance between the mobile domain and N-helix of Tn while rigor S1 decreases the distance at low calcium. (A) Titration of $Tn_{da}$ with actin-Tm at pCa 9.0. Aliquots of actin-Tm were added to a solution containing 50 nM $Tn_{da}$ until the intensity did not change. Addition of actin-Tm decreased donor quenching showing that mobile domain moved away from the N-helix. Saturation occurred at a mole ratio of 4 ï 5 actin-Tm to troponin suggesting that not all of the troponin was functional at this concentration. (B) Titration of actin-Tm $Tn_{da}$ with rigor S1. Aliquots of S1 were added to a solution containing 50 nM of $Tn_{da}$, 0.4 μM of actin-Tm. Addition of S1 increased donor quenching showing that mobile domain moved closer to the N-helix. Saturation occurred at about 0.65 μM S1.

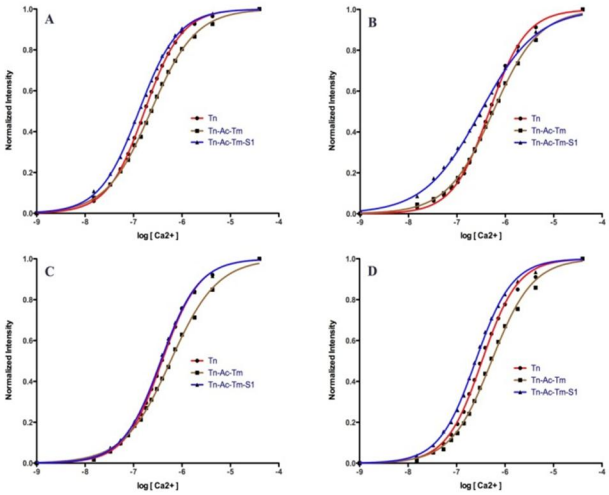


**Figure 6:** Calcium titration of Tn mutants alone, on actin-Tm, and actin-Tm-S1. The different Tn mutants are in graphs A (S-S), B (S-A), C (A-S) and D (A-A).

## CONCLUSIONS

The present results showed that not only the regulatory region but also whole TnI moves on the thin filament in response to the three states of the the thin filament. Upon $Ca^{2+}$ activation of skeletal muscle, several structural changes occur in the

troponin subunits. The present FRET measurements demonstrated that an S1-induced conformational change on thin filaments is critical for active interaction of actomyosin during the ATP cycle. The rigor binding of myosin subfragment 1(Sl) further increased these distances by 4-5 Å on mutant thin filaments. This indicates that a further conformational change on thin filaments was induced by Sl rigor-binding. The relatively large change in donor quenching of this visible spectrum probe makes it a good candidate for in situ studies on Tn subunit regulatory domain interactions within the myofilament lattice. Labeled Tn complex is stable and does not dissociate when unlabeled cysteines are substituted with Alanine, which makes it useful to observe regulatory interactions under fluorescence microscope. Figure 5,6 show that the FRET $Ca^{2+}$ titration measurements indicated that different structural regions of thin filament have different sensitivity to $Ca^{2+}$ binding to the regulatory site of sTnC.

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