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# Smooth muscle α-tropomyosin crosslinks to caldesmon, to actin and to myosin subfragment 1 on the muscle thin filament

Nina L. Golitsina[a], Sherwin S. Lehrer[a,b,*]

[a] *Muscle Research Group, Boston Biomedical Research Institute, Boston, MA 02114, USA*
[b] *Department of Neurology, Harvard Medical School, Boston, MA 02115, USA*

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**Abstract To obtain proximity information between tropomyosin (Tm) and caldesmon (CaD) on the muscle thin filament, we cloned gizzard αTm and created two single Cys mutants S56C/C190S (56Tm) and D100C/C190S (100Tm). They were labeled with benzophenone maleimide (BPM) and UV-irradiated on thin filaments. One chain of BPM-56Tm and two chains of BPM-100Tm crosslinked to CaD. Only BPM-100Tm crosslinked to actin in the absence and presence of CaD and binding of low ratios of myosin subfragment 1 (S1) prevented the crosslinking. Tm-S1 crosslinks were produced when actin·Tm was saturated with S1. Thus, CaD on the actin·Tm filament is located $<10$ Å away from Tm amino acids 56 and 100; in the closed state of the actin·Tm filament, Tm residue 100 is located close to the actin surface and is moved further away in the S1-induced open state; in the open state, S1 binds close to Tm.**



*Key words:* Muscle thin filament; Smooth muscle tropomyosin Cys mutant; Caldesmon; Actin; UV crosslinking

## 1. Introduction

Smooth muscle contraction is primarily regulated by $Ca^{2+}$ calmodulin dependent myosin light chain phosphorylation. However, there is also evidence for thin filament regulation because the actin binding protein, caldesmon (CaD), inhibits acto-myosin ATPase and tropomyosin (Tm) facilitates the inhibition [1–7]. Chalovich and colleagues suggested that CaD inhibits ATPase by blocking myosin-actin binding [8] while Marston and colleagues proposed that CaD acts by inhibiting the off to on transition between the thin filament activity states [6,9].

CaD is a flexible 74 nm long, 1.7 nm wide molecule [10,11], which covers about 10–14 actin subunits on the actin·Tm filament [12,13]. Several Tm-CaD interaction sites have been suggested [12,14–16]. However, close contact CaD with Tm on actin·Tm filaments was not seen using electron microscopy image reconstruction [17].

To further characterize the Tm-CaD interaction on actin we cloned gizzard αTm, created two Cys double mutants, 56Tm and 100Tm, with only one Cys in each located on the outside of the coiled-coil and a Ser at position 190. The mutants were labeled with benzophenone-4-maleimide (BPM), which on UV irradiation, produces a 10 Å crosslink. The proximity between smooth muscle proteins and BPM labeled Tm (BPM-Tm) was studied by noting crosslink formation on SDS-polyacrylamide gels after UV irradiation. We found that one chain of 56Tm and two chains of 100Tm were linked to CaD. Thus, CaD is located within 10 Å from Tm amino acid positions 56 and 100 on the thin filament.

We also found that 100Tm can be crosslinked to actin, and that at low myosin subfragment 1 (S1) to actin binding ratios, the crosslinking was prevented. This is direct evidence for S1-induced movement of Tm on actin from the closed to the open state [18]. In addition, we found that S1 bound to actin·Tm also could be crosslinked to 100Tm. This shows that the S1 rigor binding site on actin is close to Tm.

## 2. Materials and methods

### 2.1. DNA construction

Total RNA was isolated from chicken gizzard tissue using an RNA isolation kit (Stratagene). Gizzard cDNA was synthesized with a 'Super Script Pre-amplification System for First Strand Synthesis kit' (GIBCO BRL), and the αTm gene was PCR-amplified using Vent polymerase (New England Biolabs) with primers from 5′ to 3′: CTA-ACC-CAC-CGC-CCA-TAT-GGA-TGC-CAT-C and TGC-TGC-TAA-GAA-GGA-ATT-CAC-ATG-TTG-TTT-AAC-TCC-AG-T, covering the entire coding region plus some of the non-translated regions based on the chicken Tm gene (Izant, J.G.; GenBank M36337) and smooth 9d exon (Lemonnier, M.; GenBank X57996) sequences. Primers included *Nde*I or *Eco*RI restriction sites for cloning. The PCR product was purified by agarose gel electrophoresis, cloned before sequencing, into a purified PAED4 plasmid at the *Nde*I and *Eco*RI sites and transformed into *E. coli* (strain HB101 or NM522) for amplification. The amplified plasmid with gizzard smooth αTm gene was purified (QIAGEN Plasmid Midi kit) and used for sequencing (Sequenase Version 2.0 DNA Sequencing kit (USB), or Tufts University sequencing facility), and transformed into BL21(DE3)pLysS *E. coli* for expression. The Cys free mutant Cys190Ser αTm was created with the QuikChange Site-Directed Mutagenesis kit (Stratagene) and, after sequencing, was used to produce 56Tm and 100Tm mutants. The following oligonucleotide primers were used for mutagenesis (sequences given for non-coding strand in 5′ to 3′ orientation; the bold font indicates the site of the mutation): GCT-CAG-C**AG**-**A**TT-TGC-TTT-C (position 190); CAG-CAC-TTG-GTC-CCT-**GCA**-ATC-CTC-CGT-CAC (position 56); CTG-AGC-CCG-**ACA**-CAA-TTC-TTC (position 100). Both double mutants were sequenced to verify the mutation positions, the absence of spontaneous mutation, and used for expression in BL21(DE3)-pLysS *E. coli* under induction by isopropyl β-D-thiogalactopyranoside (IPTG).

### 2.2. Purification of recombinant GTms

The cell pellets were defrosted in 50 ml 50 mM Tris-HCl pH 7.7,

*Corresponding author. Fax: (1)-617-912 0308.
E-mail: lehrer@bbri.org

*Abbreviations:* CaD, caldesmon; Tm, tropomyosin; 56Tm and 100Tm, Tm double mutant C190S/S56C and C190S/D100C, respectively; S1, myosin subfragment 1; NEM-S1, *N*-ethylmaleimide treated S1; BPM, benzophenone-4-maleimide; BPM-Tm, BPM labeled Tm


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100 mM KCl, 5 mM EDTA, 5 mM DTT per pellet with 5 ml/20 g cell of a bacterial cell protease inhibitor cocktail (Sigma) and homogenized by flow pipetting for 30 min. The KCl concentration was increased to 300 mM, the homogenate was incubated for 1 h on ice and centrifuged 1 h at 50 000×*g*, 4°C. The supernatant, containing recombinant Tm was treated with DNase and purified by ammonium sulfate (35–70%) fractionation, isoelectric precipitation at pH 4.7, FPLC (Mono Q, Pharmacia and hydroxyapatite, Bio-Rad columns), dialyzed and lyophilized. About 100 mg recombinant Tm was purified from 1.5 l cell suspension. The N-terminal amino acid sequence and the CD spectrum of the Tm mutants agreed with native gizzard Tm. The Tm mutants inhibited actin-activated S1 ATPase activity at low S1/actin ratio, as expected [19,20].

### 2.3. Binding to F-actin

Increasing concentrations of the Tms (clarified for 60 min by centrifugation at 150 000×*g*) were incubated with 23.2 μM F-actin in 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, 30 mM HEPES pH 7.5 for 1 h and centrifuged at 140 000g for 30 min at 20°C. The Tm-actin pellets and supernatants were run on 12% SDS 4 M urea polyacrylamide gels. The amount of Tm bound to actin was determined by densitometry after imaging gel bands with the NIH Image program using a Kodak density step tablet for calibration. Binding constants and stoichiometry were calculated from fitting binding profiles to the quadratic binding equation [21] using the non-weighted non-linear least-squares program in Kaleidagraph (Synergy)

### 2.4. BPM labelling of Tm

All operations were performed in the dark. 56Tm and 100Tm were treated 10 mM DTT for 1 h at room temperature and extensively dialyzed to remove DTT against 30 mM Tris-HCl buffer pH 7.7, 50 mM NaCl, 1 mM EDTA. A 5× molar excess of BPM was added from 20 mM stock solution in DMF, and samples were rotated for 4 h at room temperature. The reaction was quenched with 5 mM DTT, and samples dialyzed against 20 mM Tris-HCl buffer pH 7.4, 100 mM NaCl. A Cys free Tm mutant treated at the same time showed no labeling.

### 2.5. Photo-crosslinking methods

BPM-Tms, 4 μM; actin, 28 μM; CaD, 4 μM; S1 (varied concentrations) were mixed in 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, 30 mM HEPES pH 7.5 for 30 min. UV irradiation was carried out in 5 mm diameter glass tubes in a Rayonet RPR-100 photochemical reactor equipped with 16 '3500' lamps (Southern New England Ultraviolet, Hamden, CT, USA) at 4°C for 20 min and thin filaments were centrifuged at 140 000×*g* for 30 min at 20°C. The crosslinked products were analyzed in pellets and supernatants with 4–20% gradient SDS-polyacrylamide gels (Novex). The apparent molecular weight of the gel bands were calculated using the mobility of the molecular weight markers (Bio-Rad) on the same gel as standards. Densitometric analyses was done after imaging gel bands with the NIH Image program.

Rabbit skeletal actin was purified from a muscle acetone powder [22]. S1 was prepared by chymotryptic digestion of myosin [23]. *N*-ethylmaleimide treated S1 (NEM-S1) was prepared according to Williams et al. [24]. CaD was supplied by Dr. C.-L.A. Wang. Protein concentrations were determined by UV spectrophotometry using the following absorbance values $(mg/ml)^{-1}cm^{-1}$ and molecular weights (Da): G-actin, 0.63 at 290 nm and 43 000; Tm, 0.2 at 277 nm and 66 000; S1, 0.74 at 280 nm and 120 000; CaD, 0.325 at 280 and 89 000. The concentration of the labeled Tms was determined by the BCA-Protein assay (Pierce), using unlabeled Tm as a standard. BPM concentration was calculated using $\varepsilon_{280} = 13\,000\ M^{-1}cm^{-1}$ [25] after subtracting the protein contribution from the measured total absorbance of the sample. The labeling ratios for BPM-Tms were between 70 and 96%.

## 3. Results

### 3.1. Binding of E. coli expressed ααTms to F-actin.

The binding of the recombinant Tms to actin was studied by ultracentrifugation and densitometry of SDS polyacrylamide gels containing urea (Section 2.3). Addition of urea helps to separate the 33 kDa gizzard αTm monomer from actin which comigrate in normal SDS-polyacrylamide gels (Fig. 1 and 2). An approximate binding constant of 0.2–$0.5\times10^6\ M^{-1}$ at 100 mM NaCl, 2 mM $Mg^{2+}$, pH 7.5 was obtained for wild-type ααTm and both mutants. The binding of recombinant Tm expressed in *E. coli* is weaker than native muscle ααTm due to the lack of acetylation at the N-terminus in agreement with other work [26]. However, a sufficient amount of gizzard recombinant Tm was bound to identify crosslinked species that were produced upon UV irradiation (see below).

### 3.2. Crosslinking of BPM-Tms in complexes with actin and actin·CaD.

UV irradiation produced some amount of interchain crosslinks species for both Tm mutants, more for BPM-56Tm both in the absence and presence of actin (compare Fig. 1A, lanes 1 and 2 with Fig. 1B, lanes 1 and 2). Note that the αTm chain

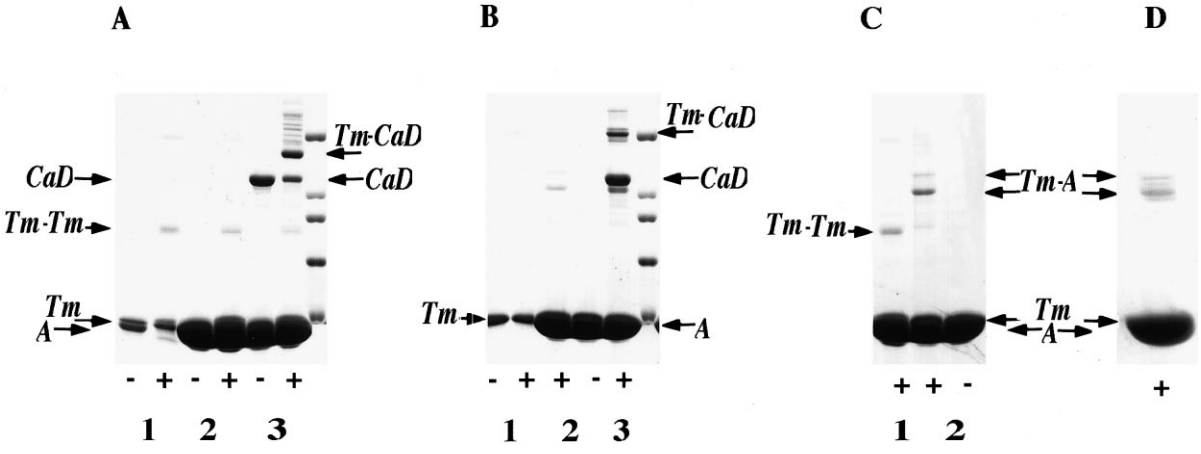


Fig. 1. A,B,C: Crosslinking of BPM-56 and BPM-100 Tms to CaD and BPM-100Tm to actin. Gel bands (equal loading) of the samples before (−) and after (+) UV irradiation and after pelleting of the thin filaments. A: BPM-56Tm-CaD crosslinked species. Lanes 1, BPM-56Tm; lanes 2, BPM-56Tm+actin; lanes 3, BPM-56Tm+actin+CaD. B: BPM-100Tm-CaD crosslinked species. Lanes 1, BPM-100Tm; lanes 2, BPM-100Tm+actin; lane 3, BPM-100Tm+actin+CaD. C: BPM-100Tm-actin crosslinked species. Lane 1, BPM-56Tm+actin; lanes 2, BPM-100Tm+actin. D: Fluorescence of actin containing species after UV irradiation of BPM-100Tm+acrylodan-actin. Details in Section 2.5. Molecular weight markers shown on un-numbered lines on (A) and (B) are in kDa from top to bottom: 200; 116.5; 97.4; 66.2; 45.

migrates abnormally on SDS-gels with an apparent monomer molecular weight of 44 kDa instead of the actual 33 kDa and the crosslinked dimer migrates as a 88 kDa species (2×44 kDa). BPM-56Tm has a greater mobility than unlabeled 56Tm, resulting in two monomer bands in this incompletely labeled protein (Fig. 1A, lanes 1). Densitometric analysis of these bands indicated that 56Tm was labeled to about 70%.

BPM-100Tm forms crosslinks to actin both in the absence and presence of CaD (Fig. 1B, lanes 2+, 3; Fig. 1C, lane 2+). In contrast, BPM-56Tm does not crosslink to actin (Fig. 1A, lane 2+). The presence of actin in the 100Tm-actin crosslink was verified by a control study with acrylodan-labeled actin which showed fluorescence in the gel at the position of the crosslinked species (Fig. 1D). The apparent molecular weight of the main Tm-actin crosslink was 126 kDa, indicating that the species probably consisted of two Tm chains linked to one actin chain (88+43 kDa).

When CaD was bound to actin·BPM-Tm, crosslinks between BPM-Tm and CaD were observed for both 56Tm and 100Tm (Fig. 1A,B, lanes 3). The apparent molecular weight of the main crosslinked species for 56Tm-CaD and for 100Tm-CaD was 162 and 204 kDa, respectively, indicating that one chain of 56Tm (44 kDa) and two chains of 100Tm (88 kDa) were linked to CaD (135 kDa). The actual molecular weight of the main CaD-BPM-56Tm crosslinked species was determined by mass spectral analysis to be 122.7 kDa (33 kDa +89 kDa) (data not shown). More than 50% of the CaD was crosslinked to 56Tm and about 30% to 100Tm. All crosslinked species remained bound to actin as indicated by actin sedimentation. The presence of small amounts of high molecular weight bands above the CaD-Tm main band is probably due to non-specific crosslinking.

### 3.3. S1 inhibits crosslinking of 100Tm to actin at low binding ratios and crosslinks to 100Tm at high binding ratios

The influence of S1 binding on the crosslinking between BPM-100Tm and actin was studied to determine if there are changes in the crosslinking pattern when the actin·Tm filament is shifted from the closed to the open state by rigor-like S1-actin bonds [18]. Both NEM-S1 and S1 were used to form the rigor bonds. Binding of S1 or NEM-S1 at low ratios to actin inhibited crosslinking of Tm to actin (Fig. 2). At

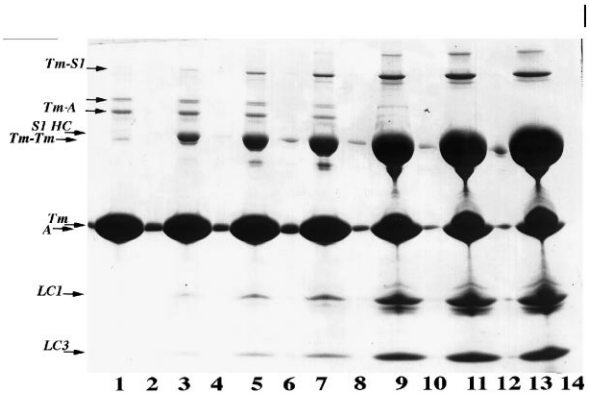


Fig. 2. The influence of S1 binding to actin·Tm on crosslinking of BPM-100Tm to actin and S1. Gradient (4–20%) SDS-polyacrylamide gel of BPM-100Tm+actin pellets containing increasing amounts of NEM-S1 after UV irradiation. Lanes 1, 3, 5, 7, 9, 11, 13; NEM-S1: 0 μM, 1 μM, 2 μM, 4 μM, 16 μM, 28 μM and 45.2 μM, respectively.

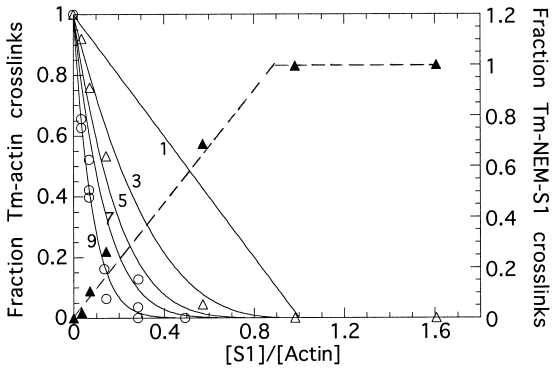


Fig. 3. Loss of Tm-actin crosslinks and production of Tm-S1 crosslinks by S1 binding to BPM-Tm·actin. Tm-actin crosslinks from two independent experiments with S1(○) and one with NEM-S1 (△). Tm-NEM-S1 crosslinks, (▲). Data obtained from densitometry of major crosslinked band for BPM-Tm·actin or Tm-NEM-S1. Conditions in Section 2. Solid curves, $f_{XL} = (1-f_b)^n$ for $n = 1, 3, 5, 7, 9$. $f_{XL}$ = fraction Tm-actin crosslinks, $f_b$ = fraction S1 bound to actin. Dashed line: fraction Tm-NEM-S1 crosslinks, drawn by hand.

higher ratios of S1 to actin, new crosslinked Tm-S1 species were observed with an apparent molecular weight of 180 kDa for the main band, indicating that crosslinks were formed between Tm dimer and S1 heavy chain (88+90 kDa).

The S1 dependence of the formation of Tm-actin crosslinked bands is shown in Fig. 3 (open symbols). The solid lines show curves plotted according to $f_{XL} = (1-f_b)^n$, where $n$ is the apparent cooperative unit size for $n = 1, 3, 5, 7$ and 9. It appears that within experimental error, a value of $n \geq 7$ best describes the S1-dependence. Thus the random binding of one S1 to seven actin subunits is sufficient to shift the Tm to a state where crosslinks do not readily take place. Since at the concentrations used, the recombinant Tm was not completely bound to actin in the absence of S1, and S1 induces Tm binding [27,28], somewhat less S1 may be sufficient to inhibit crosslinking.

In contrast to the loss of Tm-actin crosslinking at low S1/actin ratios, Tm-S1 crosslinks were produced at higher ratios of S1/actin which formed in proportion to S1 binding.

## 4. Discussion

### 4.1. Tm-CaD crosslink

This crosslinking study has shown that Tm amino acid residues 56 and 100, which are 1/5 and 1/3 from the N-terminus, respectively, are within 10A of CaD on the actin·Tm·CaD thin filament. More then 50% of the CaD was crosslinked to BPM-56Tm to produce crosslinked species of 162 kDa apparent molecular weight (on gels) or 123 kDa (actual), indicating that one Tm chain of 33 kDa was linked to CaD (89 kDa). In contrast, the CaD-100Tm crosslinked product (204 kDa apparent molecular weight) was composed of two Tm chains and one CaD. This can reflect a different arrangement of CaD molecule on the thin filament in the 56 and 100 Tm region. CaD may locate close to only one of the two Tm chains near position 56, but close to both chains near position 100. Other explanations are possible but identification of the crosslinked sites will clarify the arrangement (work in progress).

Previous studies have indicated several regions of CaD interaction with Tm alone and on actin [14,16]. Electron micros-

copy image reconstruction of smooth muscle thin filaments did not show contacts between Tm and CaD, although 'occasional' contacts were not ruled out [29]. Our data suggest that CaD binds to the thin filament within 10 Å of Tm near position 56 and 100. This may be missed by the helical averaging used for image reconstruction. Further crosslinking studies with mutants in the C-terminal half of Tm will help understand if these contacts are more extensive.

### 4.2. Tm-actin crosslink

Although both BPM-labeled Tms could be crosslinked to CaD, only BPM-100Tm had a significant tendency to crosslink to actin. This may be due to the more exposed location of the label at position (b) for 100 Tm compared to (g) for 56 Tm in the Tm seven residue helical repeat (a–g). Two actin-100Tm crosslinked products, (Fig. 1C, lane 2+) were produced due to the formation of two different actin-ααTm crosslinked species involving two Tm chains: a crosslinked Tm dimer crosslinked to actin, ($_{\alpha}$Tm-$_{\alpha}$Tm)-actin or each Tm chain crosslinked to actin independently, $_{\alpha}$Tm-actin-$_{\alpha}$Tm, where $_{\alpha}$Tm represents one chain of Tm molecule.

In the presence of CaD a greater yield of Tm-actin crosslinks was obtained (compare Fig. 1B lane 3+ and 2+), most probably due to the increased binding of Tm to actin caused by CaD. An alternate explanation for the increased yield may be the effect of CaD on position of Tm on actin since electron microscopy image reconstruction indicated a different location of Tm due to CaD binding [29].

The crosslinked species at 88 kDa apparent molecular weight that is produced on irradiation of BPM-56Tm·actin appears to be an αTm-αTm dimer. Although the apparent molecular weight of an αTm-actin crosslinked species is similar, the production of this species appears unlikely because no acrylodan fluorescence from labeled actin was seen in this region of the gel on UV irradiation of BPM-56Tm·actin.

### 4.3. S1 effects on Tm crosslinking pattern in thin filament

The binding of S1 to actin·Tm affected the crosslinking pattern in two ways: (1) at low binding ratios, S1 inhibited the ability of BPM-Tm to crosslink to actin; (2) at higher binding ratios, S1 itself crosslinked to BPM-Tm. These data can be explained by the two-state model of the actin·Tm filament in which skeletal and smooth Tm on actin are predominantly in the closed state [18]. The binding of S1 at low ratios, however, can trap Tm in the open state [30–33]. The plot of the loss of Tm-actin crosslinks vs. S1/actin is similar to the dependence seen earlier with fluorescence probes that monitor the closed/open transition [28,33,34].

A cooperative unit size of $n \geq 7$ was considered as a best fit for the data in Fig. 3; (solid line) in agreement with value of $n$ obtained with pyrene labeled wild-type recombinant gizzard and rat smooth ααTm (unpublished). Thus, these data provide direct evidence for S1-induced Tm movement on actin whereby an average binding of one S1 to seven actin subunits can induce Tm movement to open at least seven actin subunits.

At higher S1/actin ratios, S1-Tm crosslinked species were also observed (Fig. 2) whose formation was directly proportional to the binding of S1 to actin, suggesting that S1 on one of the subunits of actin is close to the BPM label on 100Tm. This is evidence for the close ($< 10$ Å) proximity of Tm and S1 in the S1-actin·Tm rigor complex in the filament open state. A previous crosslinking study also obtained evidence for S1 binding on actin·Tm near amino acid 190 of skeletal Tm although the yield was quite low [35].

Recent independent data by Monteiro and Reinach using skeletal Tm showed that Tm-actin crosslinks could be formed at several positions on Tm including 100 (personal communication). They also showed that S1-binding inhibited crosslink formation for actin·Tm·troponin, independent of $Ca^{2+}$, despite the different source of Tm. Another study showed changes in fluorescence energy transfer between smooth Tm and actin on binding S1 at low ratios [36]. These observations are in general agreement with our work.

In conclusion, our results indicate that: (1) CaD on the actin·Tm thin filament is located less then 10 Å from Tm amino acid residues 56 and 100; (2) In the closed state Tm amino acid 100 is within 10 Å of the actin and is farther then 10 Å in the S1-induced open state; (3) S1 bound to actin·Tm is located less than 10 Å from Tm near amino acid 100.

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