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Ca²⁺-dependent photocrosslinking of tropomyosin residue 146 to residues 157-163 in the C-terminal domain of troponin I in reconstituted skeletal muscle thin filaments

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Summary

The Ca²⁺-dependent interaction of troponin I (TnI) with actin•tropomyosin (Actin•Tm) in the muscle thin filament is a critical step in the regulation of muscle contraction. Previous studies have suggested that, in the absence of Ca²⁺, TnI interacts with Tm as well as actin in the reconstituted muscle thin filament, maintaining Tm at the outer domain of actin and blocking myosin-actin interaction. To obtain direct evidence for this Tm-TnI interaction we performed photochemical crosslinking studies using Tm labeled with 4-maleimidobenzophenone (BPmal) at position 146 or 174 (Tm146* or Tm174*, respectively), reconstituted with actin and troponin (composed of TnI; troponin T, TnT; and troponin C, TnC) or with actin and TnI. After near uv-irradiation, SDS gels of the Tm*146-containing thin filament showed 3 new high molecular weight bands determined to be crosslinked products Tm*146-TnI, Tm*146-TnC and Tm*146-TnT using fluorescence-labeled TnI, mass spectrometry and Western blots. While Tm*146-TnI was produced only in the absence of Ca²⁺, the production of the other crosslinked species did not show a Ca²⁺ dependence. Tm*174 mainly crosslinked to TnT. In the absence of actin a similar crosslinking pattern was obtained with a much lower yield. A tryptic peptide from Tm*146-TnI of MW 2601.2 Da that was not present in the tryptic peptides of Tm*146 or TnI was identified using HPLC and MALDI-TOF. This was shown, using absorption and fluorescence spectroscopy, to be the BPmal-labeled peptide from Tm crosslinked to TnI peptide 157-163. These data showing that a region in the C-terminal domain of TnI interacts with Tm in the absence of Ca²⁺ support the hypothesis that a TnI-Tm interaction maintains Tm at the outer domain of actin, and will help efforts to localize Tn in the actin•Tm muscle thin filament.

Keywords

striated muscle; tropomyosin; troponin; calcium regulation; photocrosslinking; benzophenone

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Introduction

Contraction of striated muscle is triggered by Ca^{2+} and regulated via the thin filament proteins troponin (Tn) and tropomyosin (Tm).¹ Tn is composed of the Ca^{2+} -binding, inhibitory and Tm-binding subunits (TnC, TnI and TnT respectively), with a stoichiometry of 1:1:1. Tm is an α -helical coiled-coil that interacts with actin along the entire length of the F-actin filament. The stoichiometry of the thin filament is 7:1:1 actin:Tm:Tn. Extensive biochemical and biophysical studies have revealed the following picture for thin filament regulation: the thin filament can equilibrate between three functional states (Blocked-Closed-Open) as influenced by Ca^{2+} and myosin subfragment-1 (S1). The three states have been shown to be associated with 3 Tm positions in the actin filament.² Depending on the state and its corresponding position, Tm sterically regulates myosin head binding to actin.³ The “Blocked” state predominates in the absence of Ca^{2+} . TnC’s N-terminal regulatory hydrophobic pocket is closed and the actin-binding segments of TnI, the inhibitory region (residues 96-116) and a C-terminal region (residues 133-150), interact with actin at its outer domain.⁴ Tm is positioned at the outer domain of actin where it blocks the myosin-binding sites on actin. Consequently, contraction is inhibited. The C-terminal domain of TnI is also known to be involved in inhibition of actin•S1 ATPase (see review of Szczesna and Potter⁵). The “Closed” state predominates when 2 Ca^{2+} are bound at the Ca^{2+} -specific sites of TnC; TnC’s N-terminal hydrophobic pocket is open and the inhibitory and C-terminal regions of TnI dissociate from actin and interact with TnC. Tm moves to a region between the outer and inner domains of actin, exposing myosin binding sites in actin and permitting the initial, weak binding of myosin crossbridges to actin. In the “Open” state myosin crossbridges cooperatively move Tm further into actin’s inner domain, exposing the remaining myosin binding sites and permitting strong binding, or force-generating, myosin crossbridges to bind actin.

Over the years many aspects of this picture have been clarified, with the notable exceptions of the following: 1) How and where is the Tn complex localized along the actin•Tm thin filament? 2) How does the binding of TnI’s actin binding regions to actin give rise to the movement of Tm to the outer domain of actin? With respect to the link between binding of TnI to the thin filament and the movement of Tm to the blocking position, it was first proposed by Potter and Gergely⁶ that TnI interacts with Tm as well as actin, so that binding of TnI at the outer domain of actin helps to “lock” Tm at its blocking position when Ca^{2+} is absent. Indirect evidence for this interaction was obtained from our studies on the interaction between TnI and actin in the absence and presence of Tm,^{7; 8} and from our finding that TnI crosslinks to regions of actin that are near the putative Tm-binding site,⁴ but direct evidence was still lacking.

For a full understanding of the mechanism for thin filament regulation, it would clearly be ideal to have structural information on the localization of the Tn complex as well as that of Tm in the F-actin filament. In this respect, high resolution structures of the Tn complex (in the absence of actin and Tm) have been obtained in the absence and presence of Ca^{2+} , but the C-terminal domain of TnI (amino acid sequence 144-182 or 188-210 for rabbit skeletal or cardiac, respectively, was not resolved^{9; 10} due to its unstructured state.¹¹ Attempts have been made to dock the atomic structure of Tn onto the EM reconstruction-derived structure of actin•Tm. The resultant structure showed an additional Tn domain interacting with actin only in the absence of Ca^{2+} .^{12; 13} However, an interaction of this additional domain with Tm was not observed and it was not possible to dock the Tn complex at a specific site along the Tm molecule with helical-reconstruction modeling, in which Tm is treated as a uniform rod. Clearly, information on the sites of contact between Tn and Tm will advance this docking procedure.

To examine whether Tm makes contact with TnI in the thin filament and, if so, determine the sites of contact between them, we prepared 2 single site Cys mutants of chicken skeletal Tm, I146C and G174C. Both contained an Ala-Ser dipeptide at the N-terminus to restore actin

binding that would otherwise be lost due to the lack of N-terminal acetylation.¹⁴ The rationale for choosing those positions was based on: 1) indications from earlier work that Tn interacted between the middle of Tm (~residue 142) and the region near Cys190;¹⁵ 2) Our FRET studies which indicated that the distance between Cys 190 and several sites on TnI were >60Å (unpublished data); 3) the exposure of residues 146 and 174 - they are f-residues on the outside of the 7-residue repeat of a coiled coil (a-g). We labeled each mutant with BPmal and photolyzed the reconstituted thin filament containing each labeled Tm (Tm*146 or Tm*174). We found that Tm*146 crosslinked to TnI, TnT and TnC and that Tm*174 crosslinked to TnT, and weakly to TnC and TnI. However, only the Tm*146-TnI crosslink was sensitive to Ca^{2+} - it only formed in the absence of Ca^{2+} . These data indicate that a part of TnI interacts near Tm residue 146 in a Ca^{2+} -dependent manner. Furthermore, the region of TnI that crosslinked to Tm*146 was determined to be within residues 157-163 in the C-terminal domain. Our findings provide direct evidence that Tm interacts with TnI in a manner that is consistent with the early proposed model for thin filament regulation.⁶

Results

Identification of crosslinked products of BPmal-labeled Tm in reconstituted thin filaments

Tm*174 or Tm*146 was separately photolyzed in reconstituted thin filament complexes containing actin, Tm* and Tn (actin•Tm*•Tn), and run on SDS gels (Fig. 1) The main crosslinked species for Tm*174 was with TnT (Fig. 1A) whose yield was independent of Ca^{2+} . The Tm*174-TnT crosslinked species was identified by anti-TnT antibody on Western blots (Fig. 2C) and by MALDI-TOF mass spectra (Table 1). Minor crosslinked bands insensitive to Ca^{2+} were also observed and were tentatively identified as Tm*174-TnI and Tm*174-TnC from their gel mobilities and apparent molecular weights.

The most interesting finding was that Tm*146 crosslinked to TnI in a Ca^{2+} -dependent manner (Fig. 1, B & C). The crosslinked species labeled Tm*146-TnI was identified with the fluorescence of a TnI that was labeled with tetramethylrhodamine (TnI-TMR) at Cys 133 (Fig. 1C). Crosslinks were formed in the absence of Ca^{2+} (Fig. 1C -) but not in its presence (1C +). A band corresponding to crosslinking between Tm*146 and TnI was also seen when a complex with actin, Tm* and TnI (actin•Tm*•TnI, Fig. 1D) was photolyzed. This species was also identified as Tm*146-TnI with the use of TnI-TMR (data not shown). The less intense band above the main crosslinked band in Fig. 1D had a mobility consistent with TnI-Tm-TnI, a strong possibility in view of a small but significant fraction of Tms having a label on each chain in the same position. No crosslinks were formed between Tm* and actin in photolyzed actin•Tm*•Tn indicated by the lack of fluorescent species above the actin gel band, with the use of actin labeled with the fluorescent probe 1,5-IAEDANS (data not shown). The band labeled Tm-TnC was identified by its Ca^{2+} -dependent mobility as we will later show in Fig. 3. When Tm*146 was photolyzed in a complex with Tn (absence of actin), 3 gel bands were observed with the same mobilities as the crosslinked bands seen for actin•Tm*•Tn, but with much reduced yields (Fig. 2). Thus, a Tm*-TnI crosslink was produced in the absence of actin, with a yield that had some Ca^{2+} -dependence.

The crosslink between Tm*146 and TnC was identified by the known shift to faster mobility in SDS gels that occurs upon Ca^{2+} binding to TnC¹⁶ (Fig. 3A + compared to Fig. 3A -). This shift can be expected to occur for the crosslinked species, Tm*146-TnC, as well. Samples which were photolyzed in the absence and in the presence of Ca^{2+} and run as such (Fig 3A) were also run in the presence of EDTA, which removes the Ca^{2+} from TnC (Fig. 3B). It is seen that in the presence of EDTA, the Ca^{2+} -dependent mobility shift was abolished, both for the crosslinked and uncrosslinked TnC band. The identities of all these crosslinked species were confirmed by MALDI-TOF (Table I).

Effect of myosin subfragment 1 (S1) binding to actin on Tm*146-TnI crosslinking

To determine the effect of S1 binding to actin on the Tm*146-TnI crosslink, we repeated the photolysis in the presence of increasing [S1] and in the absence of Ca^{2+} . Because the Tm-TnI crosslinked band overlapped with the S1 heavy chain band in SDS gels (Fig. 4), we used TnI labeled with TMR to monitor the effect of S1 binding. We found that the Tm*146-TnI crosslink was lost as the S1/actin ratio increased (Fig. 3B). The crosslink was lost at greater than 1/7 S1/actin in the absence of Ca^{2+} in agreement with our previous data which showed that S1 dissociates the TnC•TnI complex and TnI from actin•Tm before actin is fully saturated with S1.⁸ These data confirm that S1 locally dissociates TnI via a long-range effect associated with the movement of Tm on actin, rather than a direct competition of S1 with TnI on an actin subunit. The latter would be seen at higher ratios of S1/actin where the probability of S1 binding to TnI-bound sites on actin would be greater.

Identification of the crosslinked TnI peptide

To separate the crosslinked from uncrosslinked species, reverse phase HPLC was used with a water/acetonitrile gradient (Fig. 5). New peaks in the chromatogram were obtained after photolysis. Crosslinked species were identified by the agreement of the observed with the calculated molecular weights (Table 1) in the fractions containing the mixture noted in Fig. 1. The crosslinked species were separated on SDS-gels and the bands were subjected to in-gel trypsin digestion. Tm*146-TnI produced a peptide with a molecular weight of 2601.9 not seen in the tryptic digests of the uncrosslinked Tm and TnI control bands (Fig. 6). Three peptides with molecular weights calculated from the possible tryptic peptides containing a BPmal crosslink close to the observed value contained TnI peptides from the C-terminal domain (Table 2). However, two of the peptides, which required modified groups (oxidized Met and hydrolyzed maleimide) to agree with the determined molecular weight, seemed less probable in view of the lack of oxidation of Met and lack of hydrolysis of the maleimide group seen in other peptides. Note that the peptide comprising residues 157–163 is the only one that contains a Trp. For further analysis of this peptide a simpler photolyzed system (actin•Tm*•TnI), which contained the same Tm*146-TnI crosslinked species, was separated from actin and TnI by HPLC (C4 column). To identify the peptide that contained Trp and the benzophenone moiety, the tryptic peptides were chromatographed on a C18 reversed phase column and the eluates were monitored at 280 nm where benzophenone and Trp absorb. Fractions that showed absorption at 280 nm were collected for spectral analysis (Fig. 7(1)). Fraction B showed an absorption peak at 260 nm (Fig 7(2)), identifying it as a peptide that contained benzophenone.¹⁷ Fraction A did not have appreciable absorption in the 250–350 nm region. Peptide B contained Trp as indicated by its fluorescence spectrum (Fig 7(3)). Thus, tryptic peptide B contained both benzophenone and Trp. Since there is only one Trp at position 161 in TnI, this identified peptide B to be the TnI peptide 157 to 163, crosslinked to the Tm tryptic peptide 145 to 160 via a benzophenone link. Thus, Tm residue 146 is within 9–10 Å of a 7s residue TnI sequence located in its C-terminal domain. The shoulder at 305 nm seen in the fluorescence spectrum of B was also seen in A, and appears to come from an impurity in the solvent.

Discussion

The Tm*146-TnI crosslink

The principal finding in this work is that in the fully reconstituted muscle thin filament, benzophenone linked to Tm residue 146 can crosslink to TnI between residues 157 and 163 in the absence but not in the presence of Ca^{2+} . Since the distance between the crosslinking sites is about 9–10 Å,¹⁷ it appears that these regions are within or close to a TnI•Tm interaction site. Thus, a region in the C-terminal domain of TnI interacts near a specific region in the middle of Tm in a Ca^{2+} -sensitive manner. Early studies have shown that the TnI-actin interaction was strengthened in the presence of Tm,^{6, 18; 19} and biochemical evidence has accumulated

suggesting that the C-terminal domain of TnI is involved in Ca^{2+} regulation.⁵; 20 That there is an interaction between TnI and Tm, however, and its relevance to muscle regulation was first proposed by Potter and Gergely.⁶ More recently, we used inhibition of S1-actin ATPase activity to show that the stoichiometry of the TnI-actin changed from 1:1 to 1:7 (TnI:actin), and the association constant increased ~20-fold in the presence of Tm.⁷; 8 The stoichiometry of the TnI•TnC interaction with actin•Tm, in the absence of Ca^{2+} , was also 1:7. We also showed that the ATPase inhibition by TnI was due to formation of the Blocked state with kinetic measurements of myosin S1 binding.⁷ These findings strongly indicate that Tm provides a binding site for TnI or TnC•TnI in the F-actin filament, and implies that TnI would interact with Tm in the “Blocked” state (absence of Ca^{2+}) in the complete actin•Tm•Tn thin filament. Our results here provide direct evidence that this interaction indeed occurs. The relevance of this interaction to the mechanism of thin filament regulation was also noted by Luo et al, who found that both actin-binding regions of TnI, the inhibitory and the C-terminal regions, interact with the “DNase loop” at actin’s outer domain in the Blocked state.⁴ Thus, our previous and current results showing that, in the Blocked state, the C-terminal domain of TnI is capable of interacting with both actin and Tm provide further experimental basis for the model of thin filament regulation proposed by Potter and Gergely.⁶

There is not enough information to determine the precise region near Tm residue 146 that interacts with TnI, nor the nature of this interaction. However, our previous results indicated that the region near Asp137 is locally flexible and undergoes fluctuations that unfolds this region sufficiently to allow trypsin to cleave at Arg133.²¹ It is possible that this locally unfolded region may act as a site of interaction with the region of TnI that crosslinks to Tm near residue 146, just 9 residues (~13 Å) from Asp137.

Effect of myosin S1 on Tm*146 crosslinking

We previously found that the binding of S1 to actin•Tm•TnI (or actin•Tm•TnI•TnC in the absence of Ca^{2+}) dissociates the TnI (or TnI•TnC) from actin below S1 saturation of actin subunits.⁸ In this work we extended those results by showing that the binding of S1 to actin•Tm•Tn in the absence of Ca^{2+} inhibited the Tm*146-TnI crosslinking below the saturation of actin sites by S1. It thus appears that the S1-induced movement of Tm on actin to the Open-state, which occurs below saturation of actin sites, causes the loss of the TnI binding site on actin•Tm, in agreement with our earlier studies,²² rather than due to direct competition of an actin site for S1 with TnI that occurs in the absence of Tm.

Relationship to previous structural studies

Structural evidence indicated that the C-terminal domain of TnI interacts with actin•Tm in the absence of Ca^{2+} .¹²; 13; ²³ But until recently, no strong structural evidence indicated a direct interaction of TnI with Tm. However, new structural data derived from 3-dimensional EM helical reconstruction and modeling of actin•Tm with a bound C-terminal cardiac TnI peptide (residues 129-310) (equivalent to skeletal TnI residues 111-282), indicated that the C-terminal domain interacts with both actin and Tm.²⁴ The helical reconstructions show that the C-terminal TnI peptide bound to one strand of double-stranded actin extends and interacts with the Tm on the other strand. The observation that the C-terminal domain of TnI interacts with Tm as well as actin, supports previous and our current evidence for this interaction. From our binding studies only one strong binding site of TnI on actin•Tm was seen. The EM reconstructions with the TnI peptide on actin•Tm, however, show a TnI peptide on every actin subunit interacting with 7 different parts of Tm. Our data, which indicate a Ca^{2+} -dependence of crosslinking of Tm to TnI even in the absence of actin, suggest that in the intact thin filament system in which actin is present, TnI is capable of interacting with the Tm that is on the same F-actin strand. This difference between the structural and the crosslinking data may be due to the high TnI peptide concentrations used in the structural studies which may have caused the

occupation of weak Tm binding sites with a 20 fold weaker binding constant than the single strong binding site.⁸

Other crosslinks

It is somewhat surprising that no crosslinks were obtained to actin in these reconstituted thin filament systems. A reasonable possibility is that TnI interacting with actin and Tm, sterically interfered with the production of an actin-Tm crosslink. We plan to study possible crosslinking to actin in the absence of Tn where such crosslinking may not be blocked.

Our data show that Tm*146 also crosslinks to TnC and to TnT independent of Ca^{2+} , at a somewhat lower efficiency than crosslinking to TnI. The multiple sites can be reconciled by the fact that the crosslinker can be on two different chains of the Tm dimer. Multiple crosslinks could also be produced if the crosslinker has some mobility. Further studies locating the crosslinking sites will be needed to provide more information. The absence of Ca^{2+} -dependence of these crosslinks indicate that TnT and TnC remain close to Tm when Tm moves to a different position on actin associated with the Blocked-Closed transition. When crosslinking was done with Tm*174, 28 residues away from residue 146, Tm mainly crosslinked to TnT. Two other minor crosslinked bands were not identified. The crosslink from Tm to TnT is not surprising in view of the extensive interactions that TnT makes with the C-terminal half of Tm.^{25; 26} The locations of the Tm*174 crosslink on TnT and the Tm*146 crosslinks to TnC and TnT will further aid in the docking of the Tn complex on the actin•Tm thin filament. Determination of these locations, along with further photocrosslinking studies using additional single Cys Tm mutants are in progress in our laboratories.

Materials and Methods

Materials

These include: 4-maleimidobenzophenone (BPmal), 1,5-IAEDANS, and tetramethyl rhodamine maleimide (TMRmal) (Molecular Probes, Invitrogen Detection Technologies); mass spectroscopy grade trypsin (Promega, Madison WI); tris(2-carboxyethyl)phosphine (TCEP) and HPLC grade acetonitrile (Pierce, Rockford IL and EMD, respectively); Sinapinic acid and α -Cyano-4-hydroxycinnamic acid (CHCA) (Sigma).

Protein Preparation and characterization

Recombinant Tms I146C/C190S (Tm146) and G174C/C190S (Tm174) having an Ala-Ser N-terminal extension were prepared as follows: DNA constructs were inserted into plasmid vector pAED4 and transformed into BL21 E. coli cells.

Expression and purification of the mutant Tms were carried out as previously reported.²¹ which involved freeze-thawing the cells and heating the extract above 80 °C to precipitate unwanted proteins, adjusting the pH of the supernatant to the isoelectric point of Tm (pH 4.6) to precipitate the Tm, and removing DNA by ion exchange FPLC at neutral pH. Actin was prepared from rabbit skeletal muscle acetone powder.^{27; 28} S1 was prepared according to standard procedures^{29,30} and Tn was a gift from the labs of Drs. D. Szczesna-Cordary and J. Potter.

Protein concentrations were determined spectrometrically using $\epsilon_{276\text{nm}} = 1.52 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for Tm, $\epsilon_{280\text{nm}} = 8.88 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for S1, $\epsilon_{290\text{nm}} = 2.71 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for G-actin, $\epsilon_{280\text{nm}} = 2.69 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for Tn and $\epsilon_{280\text{nm}} = 0.83 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for TnI. The concentrations of BPmal labeled Tms were determined by the Biuret assay. All the proteins were dissolved in 10 mM HEPES, 5 mM MgCl_2 and 50 mM NaCl, pH 7.5 (MS buffer).

All the Tm mutants had the correct N-terminal sequence, similar thermal unfolding profiles and inhibited actin-S1 ATPase to a similar extent as wild type Tm.

Protein labeling

Tm146 and Tm174 were labeled with BPmal as previously described.^{17, 31} Briefly, Tm (3.8 μ M) dissolved in MS buffer, was treated with BPmal (100 mM stock solution in dimethylformamide) at a molar ratio of 5:1 (reagent:Cys) in the presence of 2 mM TCEP, pH 7.5 for 4 hrs. at room temperature. The reaction was quenched with 10-fold excess dithiothreitol over reagent, and dialyzed against MS buffer overnight at 4 °C, in the dark, to remove excess reagent. The degree of labeling was estimated both by Ellman's assay for loss of SH groups and by spectrometry. The absorbance due to the benzophenone moiety was obtained by subtracting from the measured total absorbance the absorbance due to the Tm, which was calculated from the previously determined [Tm] and $\epsilon_{276\text{nm}} = 1.518 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. The concentration of the benzophenone moiety was then calculated using $\epsilon_{280\text{nm}} = 13,000 \text{ M}^{-1}\text{cm}^{-1}$.¹⁷ The degree of labeling was about 75%. The same procedures were followed when TnI and F-actin were labeled with TMRmal and 1,5-IAEDANS, respectively, except that the amount of TMRmal and 1,5-IAEDANS used was only 3-fold in excess over exposed Cys residues to obtain a low degree of labeling.

Photocrosslinking

Photocrosslinking was carried out in a Rayonet RPR-100 Photochemical reactor equipped with 16 "3500" lamps (Southern New England Ultraviolet Co., Hamdon, CT.) as described previously.¹⁷ The thin filament proteins, which were reconstituted at a molar ratio of 8:1:1 (actin:Tm*:Tn or TnI) containing 30.4 μ M actin, were contained in Pyrex test tubes that do not transmit light shorter in wavelength than 300 nm to minimize photodegradation of the proteins.

Purification of crosslinked products

Crosslinked and uncrosslinked samples were separated by reverse-phase HPLC on a 4.6mm $\times$ 25cm Vydac 214TP54 column (C4 column). The Tms were dissolved in Solvent A (0.1% TFA in water) and eluted at a flow rate of 0.7ml/min with the following gradients of solvent B (acetonitrile in 0.1% TFA): 30-45% for 30min, followed by holding at 45% for 15min and finally 45-55% for 20min. The eluate was monitored at 260 or 280nm. Peptides from trypsin-digested Tm*146-TnI were separated by reverse-phase HPLC using a 4.6mm $\times$ 25cm Vydac 218TP54 column (C18 column) with a similar solvent system. A linear gradient from 5% B to 40% B (0.4% B/min) at a flow rate of 1ml/min was used. Absorbance was monitored at 220 and 280nm.

In-gel digestion

The method of Lahm and Langen³² was followed with some modifications. Briefly, Coomassie stain was removed by placing the gel pieces in a 50% acetonitrile/50% 50mM ammonium bicarbonate solution for 30min and dried at room temperature using a speed vac. After adding a sufficient volume of 25 mM ammonium bicarbonate and 0.001% n-octylglucoside containing 50-100 ng trypsin per 20 μ l volume to rehydrate the gel pieces to its original volume, excess 25mM ammonium bicarbonate was added to cover the pieces completely. All the samples were incubated at 37 °C overnight and centrifuged. The supernatant was subjected to MALDI-TOF analysis the following day.

Immunoblotting

To identify the Tm-TnT crosslinked band, Western blots were performed using a specific monoclonal anti-troponin T antibody (mouse IgG1 isotype, Sigma) and detected with an anti-mouse IgM secondary antibody conjugated with the fluorescent tag IRDye 800CW (Rockland).

Mass Spectrometry

MALDI-TOF mass spectrometry was performed on a PerSeptive Biosystems Voyager BioSpectrometry work station. The higher molecular weight crosslinked products (Tm*146-TnT, Tm*146-TnC and Tm*146-TnI) and other standard proteins which were separated from C4 reverse-phase HPLC (directly mixed with H₂O and 0.1% TFA) were analyzed using sinapinic acid matrix without molecular weight calibration. To identify the tryptic peptides isolated from the C18 reverse-phase HPLC, CHCA matrix was used with angiotensin as a molecular weight standard for calibration

Spectral studies

The freeze dried peptide fractions which were collected from C18 reverse-phase HPLC were dissolved in MS buffer, and adsorption or fluorescence spectra were obtained on a Cary 50 spectrophotometer or a Varian Cary Eclipse fluorometer, respectively.

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Abbreviations used

Tm	tropomyosin
Tn	troponin
TnC, TnI and TnT	troponin C, troponin I and troponin T, respectively
S1	myosin subfragment-1
BPmal	4-maleimidobenzophenone
TMRmal	tetramethylrhodamine maleimide
TnI-TMR	TMRmal-labeled TnI
Tm*146 or Tm*174	BPmal-labeled mutant Tm with a single Cys at position 146 or 174, respectively
TCEP	tris(2-carboxyethyl)phosphine
EM	electron microscopy

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Fig. 1. Products of photolysis of Tm*174 and of Tm*146 in actin•Tm•Tn. Identification of a Ca^{2+} -dependent Tm-TnI crosslink

A, Tm*174 and **B**, Tm*146; both photolyzed in actin•Tm•Tn, and in the presence of 2 mM Ca^{2+} (+) or in the presence of 2 mM EGTA (-). **C**, Tm*146 and TMR-labeled TnI photolyzed in actin•Tm•Tn-TMR in the presence of 2mM Ca^{2+} (+) or in the presence of 2 mM EGTA (-). **D**, Tm*146 photolyzed in actin•Tm•TnI. Note that Tm*146 crosslinks to TnI in actin•Tm•Tn in the absence of Ca^{2+} (B and C, -) but not in the presence of Ca^{2+} (B and C, +), and Tm*146 crosslinks to TnI in the absence of TnC and TnT (D). Conditions: 4 μM actin, 0.5 μM Tm, 0.5 μM Tn (or TnI) in 50 mM NaCl, 5 mM MgCl_2 , 10 mM Hepes buffer, pH 7.5.

Fig. 2. Crosslinking of Tm*146 with Tn in the presence and absence of actin

Tm*146•Tn or actin•Tm*146•Tn was photolyzed under the same conditions as Fig. 1. Note that crosslinked bands at similar mobilities were formed in the absence of actin as well as in its presence.

Fig. 3. Identification of Tm*146-TnC and Tm*146-TnT crosslinked bands

A and B, Photolyzed samples of Tm*146 in actin•Tm*•Tn in the absence (-) and presence of Ca²⁺ (+). **A**, no EDTA in gel; **B**, EDTA (2 mM) in gel. Note the decreased mobility of TnC and Tm*146-TnC when EDTA is present in gel. **C**, Western blot of Tm*146-TnT using TnT antibody. Note that Tm*146 crosslinks to TnC and to TnT independent of Ca²⁺. Same conditions as Fig. 1.

Fig. 4. Effect of myosin-S1 binding to actin on the Tm*146-TnI crosslink produced in the absence of Ca^{2+}

Photolysis at increasing ratios of S1/actin in the absence of Ca^{2+} and ATP, using TMR-labeled TnI in Tn. Left lane, Coomassie stained at 0 S1; Right lanes, fluorescence at increasing ratios of S1/actin. Conditions as for Fig. 1. Note the loss of crosslinks at low S1 saturation of actin.

Fig. 5. Separation of thin filament proteins before and after photolysis of actin•Tm*146•Tn using reversed phase HPLC (C4 column)

The species were identified by the molecular weights of the fractions with MALDI-TOF (see Table 1). H₂O–CH₃CN gradient in 0.1% TFA was used with increasing CH₃CN (%B). Note that Tm+Tm* is a mixture of unlabeled and labeled Tm.

Fig. 6. MALDI-TOF spectra of in-gel trypsin digests of Tm*, TnI and Tm*146-TnI

Fractions containing Tm*146-TnI from the C4 HPLC column (Fig. 4) were subjected to SDS polyacrylamide gel electrophoresis. Excised Tm*, TnI and Tm*146-TnI gel pieces were then exhaustively digested with trypsin. Note that a new peptide of 2601.9 Da was present in the crosslinked mixture (marked with an asterisk).

Fig. 7. Analysis of purified Tm*146-TnI tryptic peptides

1. Separation of trypsin digested Tm*146-TnI with reverse phase HPLC (C18 column) using a H₂O–CH₃CN gradient with increasing CH₃CN (%B) as indicated. Absorption was monitored at 280 nm. **2.** Absorption spectra of tryptic peptides (fractions A and B). Note a strong absorption at 260 nm for fraction B due to presence of benzophenone. **3.** Fluorescence emission spectra of fractions A and B compared to L-tryptophan in buffer excited at 280 nm. The presence of tryptophan and benzophenone verifies that the crosslinked peptide is B.

Table 1

Identification of crosslinked proteins from HPLC with MALDI-TOF

Uncrosslinked	Observed MW (Da)	Predicted MW (Da)
Tm146	32878	32935
TnT	30788	30750
TnI	21113	21125
TnC	18038	18007
Crosslinked	Observed MW (Da) [#]	Predicted MW (Da) [#]
Tm*146-TnT	63682	63715
Tm*146-TnI	54205	54337
Tm*146-TnC	51121	51219

[#] Includes molecular weight (MW) of BPmal.

Table 2

Observed molecular weight (MW) of tryptic peptide of Tm*146-TnI (2601.9 Da) compared to possible TnI-Tm tryptic peptides.

TnI Peptide	Predicted MW (Da) [*]	Sequence of TnI peptide
157-163	2602.4	(R) DVGDWRK(N)
176-182 [#]	2600.3	(K) KMFESES
176-182 [‡]	2602.3	(K) KMFESES

^{*} Calculated from sequence including Tm peptide 143-154.

[#] One Met residue either from TnI peptide 176-182 or from Tm* peptide 143-154 is oxidized.

[‡] BPmal ring of Tm* peptide 143-154 is hydrolyzed.