Characteristics of Troponin C Binding to the Myofibrillar Thin Filament: Extraction of Troponin C Is Not Random Along the Length of the Thin Filament

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ABSTRACT Troponin C (TnC) is the Ca^{2+}-sensing subunit of troponin responsible for initiating the cascade of events resulting in contraction of striated muscle. This protein can be readily extracted from myofibrils with low-ionic-strength EDTA-containing buffers. The properties of TnC extraction have not been characterized at the structural level, nor have the interactions of TnC with the native myofibrillar thin filament been studied. To address these issues, fluorescein-labeled TnC, in conjunction with high-resolution digital fluorescence microscopy, was used to characterize TnC binding to myofibrils and to determine the randomness of TnC extraction. Fluorescein-5-maleimide TnC (F5M TnC) retained biological activity, as evidenced by reconstitution of Ca^{2+}-dependent ATPase activity in extracted myofibrils and binding to TnI in a Ca^{2+}-sensitive manner. The binding of F5M TnC to highly extracted myofibrils at low Ca^{2+} was restricted to the overlap region under rigor conditions, and the location of binding was not influenced by F5M TnC concentration. The addition of myosin subfragment 1 to occupy all actin sites resulted in F5M TnC being bound in both the overlap and nonoverlap regions. However, very little F5M TnC was bound to myofibrils under relaxing conditions. These results suggest that strong binding of myosin heads enhances TnC binding. At high Ca^{2+}, the pattern of F5M TnC binding was concentration dependent: binding was restricted to the overlap region at low F5M TnC concentration, whereas the binding propagated into the nonoverlap region at higher levels. Analysis of fluorescence intensity showed the greatest binding of F5M TnC at high Ca^{2+} with S1, and these conditions were used to characterize partially TnC-extracted myofibrils. Comparison of partially extracted myofibrils showed that low levels of extraction were associated with greater F5M TnC being bound in the nonoverlap region than in the overlap region relative to higher levels of extraction. These results show that TnC extraction is not random along the length of the thin filament, but occurs more readily in the nonoverlap region. This observation, in conjunction with the influence of rigor heads on the pattern of F5M TnC binding, suggests that strong myosin binding to actin stabilizes TnC binding at low Ca^{2+}.

INTRODUCTION

The contraction of striated muscle is regulated by calcium at the level of the thin filament by the proteins troponin and tropomyosin (reviewed in Leavis and Gergely, 1984). These proteins bind to the actin filament in a ratio of one troponin to one tropomyosin to seven actins, which forms the structural/functional unit of the thin filament. Troponin is a trimer made of subunits called troponin T, troponin I, and troponin C; troponin C is the calcium-binding subunit of the complex. The detailed mechanism by which calcium binding to troponin C results in activation of the thin filament is not fully understood, but involves a change in the interaction between troponin I and troponin C which, through tropomyosin, allows the myosin head to interact with actin (reviewed in Grabarek et al., 1992). Skeletal troponin C has four calcium-binding sites, two with high and two with low affinities. Biochemical studies led to the conclusion that saturation of the high-affinity sites enhances the binding of troponin C to the other troponin subunits, whereas calcium binding to the low-affinity sites is involved in the regulation of contraction (Potter and Gergely, 1975; Zot and Potter, 1982). The high-affinity sites can also bind magnesium with lower affinity than calcium, and it is thought that these sites are saturated with magnesium under physiological conditions. The crystal structure of troponin C has been determined for skeletal TnC with the high-affinity sites saturated with calcium (Satyshur et al., 1988; Herzberg and James, 1988; Satyshur et al., 1994). The molecule is dumbbell-shaped, with the divalent cation binding sites in globular domains at each end of the molecule linked by a long α-helix. The low-affinity regulatory sites are located near the N-terminus, and the high-affinity structural sites are located near the C-terminus. Recent NMR studies have elucidated the solution structure of TnC with both high- and low-affinity sites saturated (Slupsky and Sykes, 1995). The main observation of these studies relative to the crystal structure is that the sequence (residues 85–94) that links the N- and C-terminal domains is highly flexible instead of being rigid, and that calcium saturation of the N-terminal domain results in exposure of a hydrophobic pocket that likely binds to part of TnI, resulting in disinhibition of contraction.

Characterization of the role of TnC in regulation of contraction in the intact myofilament lattice has employed the technique of selective TnC extraction, using low-ionic-

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strength EDTA-containing buffers. The rather selective extraction of TnC under these conditions was adapted from techniques developed to purify TnC without the use of denaturants (Cox et al., 1981). Selective extraction of TnC from myofibrils was shown to decrease calcium-dependent ATPase activity (Zot and Potter, 1982), whereas extraction from skinned fibers resulted in a decrease in maximum force (Brandt et al., 1987; Moss et al., 1985). TnC extraction also shifted the tension-pCa relationship to higher calcium levels and decreased its slope (apparent cooperativity) (Brandt et al., 1987; Moss et al., 1985). This led Brandt et al. (1987) to propose that an individual thin filament behaved in a binary fashion and that force was determined by the number of thin filaments activated. Moss et al. (1985) inferred from their extraction results that the calcium sensitivity of a functional unit within a thin filament can differ, depending on the activation level of nearest-neighbor functional units. Both studies concluded that there must be some mechanism of interunit cooperativity transmitted by TnC.

Selective TnC extraction from myofibrils has allowed for many studies in which different types of TnC were incorporated into the native thin filament. Early studies substituted cardiac TnC into skeletal fibers (Moss et al., 1986, 1991; Kerrick et al., 1985) or skeletal TnC into cardiac trabecula (Babu et al., 1987, 1988), leading to insights into how the difference in the number of regulatory calcium-binding sites influences the tension-pCa relationship. Other studies incorporated conformationally sensitive, labeled TnC into either myofibrils (Zot et al., 1986) or skinned fibers (Zot et al., 1986; Guth and Potter, 1987), allowing for measurement of changes in TnC associated with activation of the thin filament. More recent studies have employed incorporation of mutant TnC to gain further insights into the structure/function relationships in the TnC molecule (Sweeney et al., 1990; Babu et al., 1993; Sorenson et al., 1995; Szczesna et al., 1996).

A major difficulty with using TnC extraction is a lack of knowledge of the location of TnC removal. The assumption has been that the extraction occurs in a random fashion along the thin filament. This ignorance becomes particularly important when trying to relate TnC extraction data to the mechanism of cooperativity along the thin filament. Brandt et al. (1987) addressed this by using radiolabeled TnC incorporated into skinned fibers and following its extraction kinetics and found that there was a rapidly extracting component and a slowly extracting component. They inferred that the fast extracting component was associated with loss of TnC from nonspecific sites, whereas the slow component was associated with specific sites and occurred in random fashion along the thin filament. Yates et al. (1993) have recently addressed the randomness question by monitoring the degree of extraction as a function of sarcomere length in skinned fibers. They observed that extraction at longer sarcomere lengths was associated with both a greater decrement in maximum force (at the same sarcomere length) and less TnC remaining in the fiber, as measured by quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This led them to conclude that extraction was not random along the thin filament and that it was favored in the nonoverlap region. Thus there are two studies using indirect approaches that contradict each other in terms of the randomness of TnC extraction.

To address this question, we have extracted TnC from myofibrils, followed by reincorporation of a fluorescein-labeled TnC and subsequent fluorescence microscopy to determine where the fluorescein TnC bound within the myofibril. Our studies characterized the binding of fluorescent TnC to highly extracted myofibrils in terms of the influence of calcium and rigor heads on the location and approximate amount of fluorescent TnC bound. We also characterized the location and relative amount of fluorescent TnC bound in myofibrils with different degrees of native TnC extraction. These studies showed that both calcium and rigor heads influence the location and amount of fluorescent TnC bound. Analysis of partially extracted myofibrils, reconstituted with fluorescent TnC under conditions that maximized TnC binding, showed that the extraction was not random, but occurred preferentially in the nonoverlap region, as suggested by the skinned fiber studies of Yates et al. (1993).

MATERIALS AND METHODS

Protein purification

Myofibrils were prepared from rabbit psoas as described previously (Swartz et al., 1990) and stored in 50% glycerol (v/v), 75 mM KCl, 10 mM imidazole (pH 7.2), 4 mM $MgCl_2$, 4 mM EGTA, 4 mM dithiothreitol (DTT), and 1 mM NaN_3 at −20°C until used. Troponin subunits were prepared from rabbit white muscle, following the procedure of Greaser and Gergely (1971) as modified by Potter (1982). Myosin subfragment 1 (S1) was prepared by chymotryptic digestion as described by Weeds and Pope (1977), and the S1 (A1) isoform was isolated by ion-exchange chromatography on SP-Sephadex (C-50) as described by Swartz and Moss (1992).

Protein labeling

Troponin C was reduced by incubation with 10 mM DTT, 15 mM 2-mercaptoethanol in 6 M urea, 20 mM Tris-HCl (pH 8.0), 1 mM EGTA for 1 h at 25°C. Reducing agents and urea were removed by a Sephadex G-25 column equilibrated with 90 mM KCl, 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid (PIPES) (pH 7.0), 2 mM EGTA, 3 mM $CaCl_2$, 1 mM NaN_3. The reduced protein was labeled with thiol-specific conjugation reagents [fluorescein 5-maleimide (F5M) or 3-(*N*-maleimidylpropionyl)-biocytin] by incubation with 1.5 mol reagent/mol TnC for 24 h at 22°C. The reaction was quenched with excess DTT, and the protein was desalted on Sephadex G-25 equilibrated with 10 mM PIPES (pH 7.0), 1 mM DTT to remove unconjugated reagent.

Troponin C extraction

Myofibrils were washed three times with 10 volumes of pCa 9.0 rigor buffer (20 mM PIPES (pH 7.0), 4 mM EGTA, 4 mM free $MgCl_2$, 1 mM NaN_3, 1 mM DTT, 1 mg/ml protease-free bovine serum albumin (BSA) and KCl to 180 mM ionic strength) to remove glycerol. Troponin C was extracted from myofibrils using 20 mM Tris base, 5 mM H-EDTA (pH 8.0), 0.1% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, as

described by Zot and Potter (1982) with minor modifications. The myofibril pellet was resuspended in 10 volumes of extraction buffer relative to the myofibril pellet mass. Each extraction cycle was carried out for 5 min at 20°C, followed by centrifugation at 1000 × *g* for 5 min. The myofibrils were extracted a total of eight times. After extraction, the myofibrils were washed with pCa 9.0 rigor buffer.

Assays

To monitor the extent of TnC extraction, myofibrillar ATPase activity was measured at 24°C in pCa 4.0 and 9.0 buffer (for composition, see Solutions below) by determination of phosphate liberation. Myofibrils (10 μg) in pCa 4.0 or 9.0 buffer were mixed with the proper pCa buffer and ATP (2 mM, 100 μl final volume) and incubated for 5 min at pCa 4.0 or 45 min at pCa 9.0. The reaction was quenched by the addition of 20 μl of 25% trichloroacetic acid, and the protein was pelleted by centrifugation for 15 s at 13,000 × *g* in a microfuge. The supernatant was assayed for phosphate by the method of Carter and Karl (1982), adapted for microtiter plates and a microplate reader. Unextracted and extracted myofibrils were assayed in quadruplicate. The calcium-dependent ATPase activity was determined by subtraction of the pCa 9.0 activity from the pCa 4.0 activity. The ability of F5M TnC to reconstitute calcium-dependent ATPase activity in TnC-extracted myofibrils was measured by similar methods. Extracted myofibrils (5 μg) were mixed with different levels of unlabeled or F5M TnC in pCa 4.0 solution at 2 mM ATP (100 μl final) and incubated at 23°C. At fixed time points the reaction was quenched with trichloroacetic acid followed by measurement of phosphate concentration. Data were corrected for activity at pCa 4.0 without TnC and represent the mean of four determinations at each TnC level.

The extraction of TnC from myofibrils and protein purity were analyzed by SDS-PAGE using 10% acrylamide, pH 9.3 gels as described by Fritz et al. (1989).

The extent of biotinylation of TnC was determined by the 4-hydroxyazobenzene-2-carboxylic acid (HABA) assay of Green (1970), which gave values of 0.7–0.9 mol biotin/mol TnC.

The binding of TnC to TnI was measured by a solid-phase microtiter assay. Plates (Dynatech Immulon 4) were coated with 100 μl of 0.2 μM TnI in 100 mM Na_2CO_3 (pH 9.6) for 2 h, washed with IBS (150 mM NaCl, 10 mM imidazole (pH 7.5), 0.5 mM $CaCl_2$, 0.05% Tween-20), blocked with 1 mg/ml BSA in IBS for 1 h, and then washed with IBS three times. The TnI-coated plates were incubated with 100 μl TnC for 2 h in pCa 9.0 or 4.0 rigor buffer. The plates were washed four times with IBS, incubated with streptavidin horseradish peroxidase for 2 h, and then washed four times with IBS. Color was developed by using 200 μl of 50 mM Na_2HPO_4 (pH 5.0), 20 mM citric acid, 6 mM *O*-phenylenediamine, 0.03% H_2O_2. The reaction was quenched with 100 μl of 0.5 M H_2SO_4, and absorbance was measured at 490 nm in a microplate reader. A competitive assay was developed using a fixed concentration of biotinyl-TnC and different levels of unlabeled or F5M TnC. The biotinyl-TnC was at 40 nM for pCa 4.0 and at 2 μM for pCa 9.0. Data were expressed relative to the absorbance of wells with biotinyl-TnC alone and represent the mean of eight wells per data point.

Myofibrillar protein was measured by the biuret assay (Gornall et al., 1949), using BSA as the standard, and labeled TnC was measured with the bicinchoninic acid (BCA) assay, using unlabeled TnC as the standard. The interference of DTT in the BCA assay was removed by pretreatment with iodoacetimide as previously described (Hill and Straka, 1988). The extent of labeling with fluorescein was determined by measurement of F5M TnC absorbance at 490 nm in 10 mM Tris-HCl (pH 9.5), 1 mM NaN_3 and calculation of the fluorescein concentration using a molar extinction coefficient of 70,000/M-cm. The labeling ratio of the F5M used in this study was 1.01 mol fluorescein/mol TnC. The concentration of TnC and S1 was determined by absorbance at 280 nm, using mass extinction coefficients of 0.14 (Greaser and Gergely, 1971) and 0.75 ml/mg-cm (Wagner and Weeds, 1977) and molecular weights of 18,000 and 115,000, respectively.

TNC incorporation into extracted myofibrils

Fluorescein-TnC was incorporated into extracted myofibrils by incubating myofibrils (0.25 mg/ml) in pCa 4.0 or 9.0 buffer with different levels of F5M TnC (100 μl final volume) for 15 min at room temperature. The myofibrils were pelleted by centrifugation for 15 s in a microfuge, resuspended in 100 μl fresh pCa 4.0 or 9.0 buffer, plated on a coverslip, and then fixed with 3% formaldehyde in pCa 4.0 or 9.0 buffer without DTT and BSA. The coverslips were washed with rigor buffer, placed on a slide with mounting medium (75% glycerol (v/v), 75 mM KCl, 10 mM Tris-HCl (pH 8.5), 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, 1 mg *p*-phenylenediamine/ml) and sealed with nail polish. To investigate the effect of the level of thin filament saturation with myosin heads on F5M TnC incorporation, studies were done under relaxing conditions and rigor conditions with saturating levels of S1. For relaxing conditions, rigor myofibrils were pelleted by centrifugation and resuspended in pCa 9.0 buffer containing 4 mM ATP and F5M TnC. The suspension was incubated for 15 min, pelleted, resuspended in pCa 9.0 buffer with 4 mM ATP, plated on coverslips, and then fixed with 3% formaldehyde in pCa 9.0 buffer with 4 mM ATP. For maximum thin filament saturation with myosin heads, the myofibrils were mixed with a solution containing 1 μM S1 (final) and different levels of F5M TnC. The myofibrils were processed for slides as described for the rigor experiments.

Imaging and image analysis

Myofibrils were imaged using a Zeiss Axiovert TV (Thornwood, NJ) microscope equipped with a 100× oil-immersion lens (NA 1.3) and a narrow bandpass fluorescein filter. Digital images were obtained with a 12-bit intensity resolution CCD (Kodak KAF 1300 chip; Photometrics, Tucson, AZ) controlled by a Matrox board and IPLab Spectrum software (Signal Analytics, Vienna, VA) in a Macintosh 840AV computer. The images were obtained by first acquiring the phase-contrast image and then the fluorescence image. No adjustment in focus was made between collection of the phase-contrast and fluorescence images, so that the only bleaching of fluorescence that occurred was during acquisition of the fluorescein image. An exposure time of 0.8 s was used for all fluorescence imaging, so that comparisons of fluorescence intensity could be made between treatments. IPLab Spectrum software was used for image processing and analysis of fluorescence intensity image data. For visual presentation, the 12-bit myofibril images were rotated to the horizontal with bilinear interpolation, cropped such that a Z-line was in the center of the image, and normalized within the cropped image to 8 bits. This presentation of the fluorescein fluorescence images emphasizes the distribution of the fluorescence within the myofibril for each condition, not the amount of fluorescence.

To approximate the amount of F5M TnC bound under different conditions, the fluorescence intensity per sarcomere was measured. The 12-bit image was rotated to the vertical using bilinear interpolation, and a region of interest (ROI) 120 pixels wide by three or more sarcomeres long was selected with the myofibril in the center of the long axis. This ROI was pasted into a new image, and the minimum intensity of the ROI was subtracted to give the net image. The net image was enlarged twofold, and the sarcomeres were defined by pasting a 60 × 2 pixel wide stripe across the Z-line and setting it to zero, thus separating three contiguous sarcomeres of the myofibril. The net image was then segmented at intensity values greater than 10% of the net image maximum. The mean intensity of the defined segments was then measured. Sarcomeric intensity data represent the mean of 20 myofibrils per treatment measured from three contiguous sarcomeres per myofibril. To further refine the analysis, the amount of F5M TnC bound in different regions of the myofibril was measured by a ratio approach as described previously for studies with fluorescent S1 (Swartz et al., 1996). The net image was prepared as described and enlarged threefold; then the pixel position of the Z-lines of three contiguous sarcomeres was determined from the corresponding phase-contrast image. A rectangular ROI (10 × 20 pixels (0.21 × 0.42 μm)), spaced 0.21 μm from the Z-line (center to center), with the long axis perpendicular to

the long axis of the myofibril, and in the center of the myofibril, was selected, and the average fluorescence intensity value of the region was measured to yield the intensity in the nonoverlap region. The same-sized ROI was spaced 0.9 μm from the Z-line (center to center) to yield the overlap region fluorescence intensity; then the nonoverlap-to-overlap ratio was determined. The ratio was determined for six contiguous half-sarcomeres per myofibril; the data represent the mean of 20 myofibrils.

Solutions

Solutions were made at a specified ionic strength and concentration of free $CaCl_2$ and $MgCl_2$ by using the program of Fabiato (1988) and the binding constants of Godt and Lindley (1982). The pCa 9.0 buffer was 20 mM PIPES (pH 7.0), 4 mM EGTA, 4 mM $MgCl_2$ (free), 1 mM NaN_3, and KCl to 180 mM ionic strength, whereas the pCa 4.0 buffer was the same, except that $CaCl_2$ was added to give 0.1 mM free calcium.

RESULTS

The extraction of TnC from myofibrils can be monitored by loss of the TnC band from samples analyzed by SDS-PAGE and by functional assays measuring the calcium-dependent ATPase activity of myofibrils. Fig. 1 shows both a Coomassie-stained gel (Fig. 1 *A*) and a histogram of the pCa 4.0 and 9.0 ATPase activity (Fig. 1 *B*) of differentially extracted myofibrils. The myofibrils were either unextracted (0) or extracted one, two, or eight times with Tris/EDTA as described in Materials and Methods. The TnC band shows a progressive decline in the amount with the number of extractions, so that it was not readily visible after two to eight extractions. The pCa 4.0 ATPase activity showed a corresponding decline with increased extraction such that after the first extraction, the relative ATPase activity was 48% of unextracted myofibrils. Further extractions gave a 23% relative activity after two extractions and a 12% relative activity after eight extractions. Similar data have been obtained by Zot and Potter (1982) on the extraction of TnC from rigor myofibrils. The pCa 9.0 ATPase activity showed a modest increase of 25% after eight cycles of extraction relative to unextracted myofibrils. This increase in activity my have resulted from a loss of whole troponin, but this is difficult to determine from the SDS-PAGE image. The gel image and the pCa 4.0 ATPase activity data show that TnC loss from the myofibrils was associated with a loss in calcium-dependent ATPase activity.

To determine the location of TnC extraction, a fluorescent conjugate of TnC was prepared by conjugating fluorescein-5-maleimide to the sole cysteine of rabbit skeletal TnC. This conjugate was characterized in terms of its ability to reconstitute calcium-dependent ATPase activity to highly extracted myofibrils. Fig. 2 shows the calcium-dependent ATPase activity of extracted myofibrils incubated with different concentrations of either unlabeled TnC or F5M TnC. The data demonstrate that the F5M TnC reconstituted the calcium-dependent ATPase activity in a fashion similar to that of unlabeled TnC. The data were fit with a hyperbolic equation of the form activity = V_m^* [TnC]/(K_m + [TnC]), where V_m is the maximum calcium-dependent ATPase activity and K_m is the apparent Michaelis constant for the

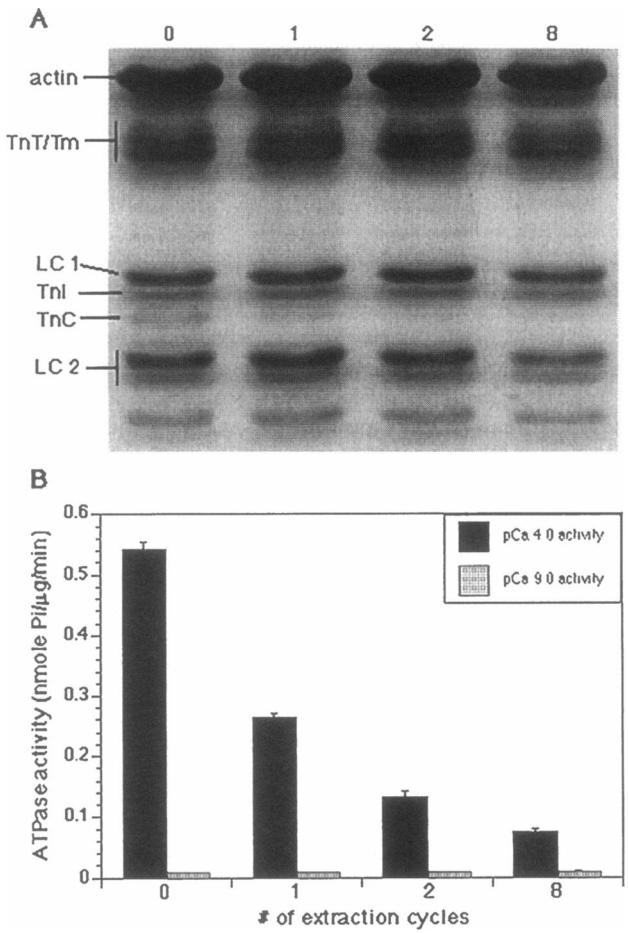

FIGURE 1 Troponin C extraction from rigor myofibrils. (*A*) Portion of a Coomassie-stained SDS-PAGE gel of control myofibrils (0) and myofibrils extracted 1, 2, or 8 times with Tris/EDTA buffer. (*B*) Histogram of the pCa 4.0 and 9.0 ATPase activity of the corresponding samples. The loss of TnC in the gel was coincident with the loss of calcium-dependent ATPase activity.

concentration of TnC for half-maximum activation. This allows for an approximation of the efficacy of F5M TnC relative to unlabeled TnC. The fitted values for unlabeled TnC were V_m = 0.40 nmol Pi/min · μg and a K_m of 18 nM, whereas the values for F5M TnC were a V_m of 0.46 nmol Pi/min · μg and a K_m of 19 nM for F5M TnC. These data suggest that F5M TnC was as good (or better) as unlabeled TnC in reconstituting calcium-dependent ATPase activity, supporting the idea that F5M TnC bound to and interacted with the components of the thin filament to activate the system in the presence of calcium. Note that the ATPase activity was measured in myofibril samples that were incubated with TnC, not myofibrils pretreated with TnC followed by washing and subsequent activity measurement.

To further characterize the interaction of F5M TnC with a component of the troponin complex, a competitive solid-phase assay was developed. Microtiter wells were coated with troponin I, followed by incubation with TnC. To monitor the amount of TnC bound, a biotin conjugate of TnC

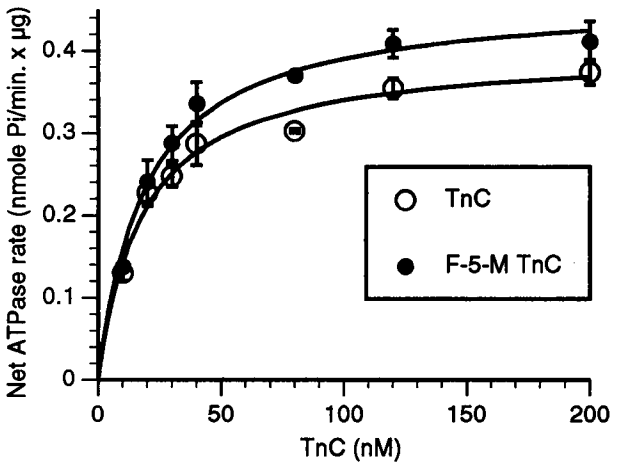

FIGURE 2 Reconstitution of calcium-activated ATPase activity in TnC-extracted myofibrils with F5M TnC. TnC-extracted myofibrils (5 μg) were mixed with different concentrations of TnC or F5M TnC in pCa 4.0 rigor buffer, ATP was added, and the reaction was quenched with TCA at different times followed by phosphate determination. Data points represent the mean (± SD, n = 4) after correction for ATPase activity at pCa 9.0. The data were fit with a hyperbolic relationship, giving a V_m of 0.40 nmol P_i/min/μg and a K_m of 18 nM for TnC, and a V_m of 0.46 nmol P_i/min/μg and a K_m of 19 nM for F5M TnC.

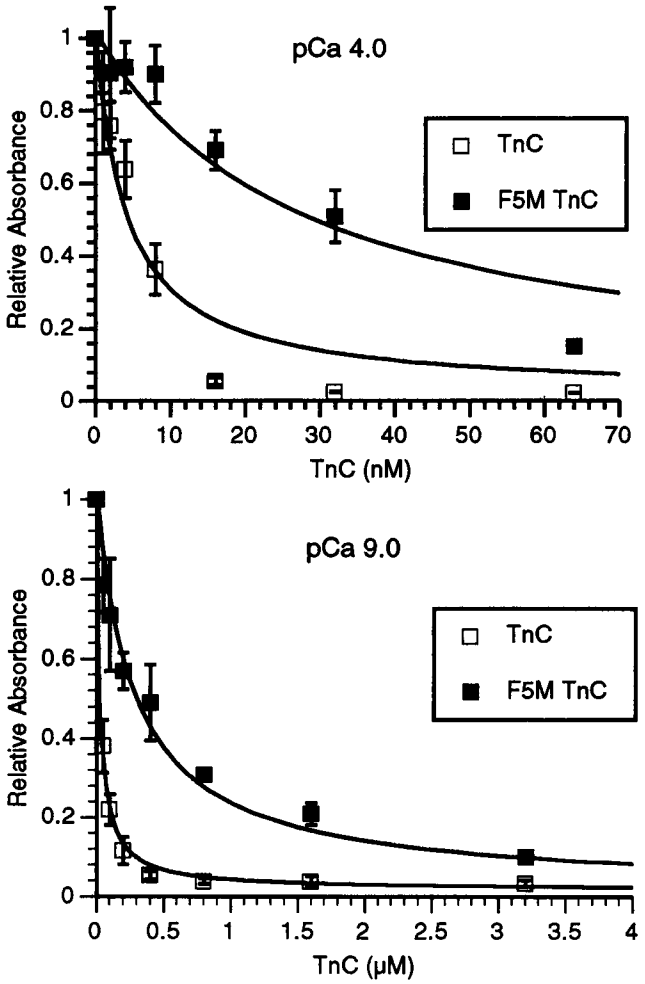

FIGURE 3 Competitive assay for TnC binding to TnI at high and low calcium. Microtiter plates were coated with TnI, washed, and then incubated with different concentrations of unlabeled TnC or F5M TnC and constant levels of biotinyl-TnC. Biotinyl-TnC concentration was 40 nM at high calcium (pCa 4.0) and 2 μM at low calcium (pCa 9.0). The wells were washed, incubated with streptavidin HRP, washed, and color developed using *O*-phenylenediamine. Data points represent the mean (± SD) of eight wells for each treatment relative to the absorbance without competing TnC. The assays show that F5M TnC binds to TnI in a calcium-sensitive manner, as does unlabeled TnC, but with a ~10-fold lower affinity.

was prepared, followed by measurement of the biotin amount in the wells, using streptavidin-horseradish peroxidase. For the competitive assay, a fixed concentration of biotin TnC was incubated in the wells with different levels of either unlabeled or F5M TnC, allowing for comparison of the ability of F5M TnC to compete for the TnC-binding sites relative to unlabeled TnC. The assay was done at both high (pCa 4.0) and low (pCa 9.0) calcium to demonstrate the calcium-sensitive nature of the TnC-TnI interaction. (A technical note: Only the incubation with the TnCs was done at the specified calcium levels; all washes and incubations with streptavidin-HRP were done at 0.1 mM calcium, to maintain the strong interaction between TnC and TnI.) Fig. 3 shows data from competitive assays at both pCa 4 (*top*) and pCa 9.0 (*bottom*). At high calcium, the data demonstrate that unlabeled TnC can readily compete with biotin-TnC for TnI binding with half-maximum inhibition occurring at ~5 nM TnC, whereas the F5M TnC reached half-maximum inhibition at ~30 nM. At low calcium, the binding of both TnC and F5M TnC was weaker than that at high calcium, with half-maximum inhibition occurring at ~50 nM for TnC and 400 nM for F5M TnC. Thus comparison of unlabeled TnC and F5M TnC shows that labeling of TnC with fluorescein weakens the interaction of TnC with TnI 5–10-fold at both high and low calcium concentrations. These data suggest that F5M TnC is a reasonable analog for the study of TnC binding to the native, myofibrillar thin filament and consequently is a tool for determining the location of TnC extraction from rigor myofibrils. This weaker binding likely results in an underestimate of the amount of F5M TnC bound to myfobrils (relative to unlabeled TnC), made by using fluorescence intensity measurements described below.

The binding of F5M TnC to rigor myofibrils was investigated at pCa 9.0 by using highly extracted myofibrils (12% relative ATPase activity) and 200 nM F5M TnC. Fig. 4 shows representative images of myofibrils treated in this manner. Myofibrils of different sarcomere lengths are compared in the montage, and they readily show that the F5M TnC bound predominantly in the A-band and more precisely, in the actin-myosin overlap region of the A-band. Two stripes of fluorescence were present in the A-band, and, with increased sarcomere length (Fig. 4, *1F–4F*), there was increased separation between the stripes. There was some low-level binding of F5M TnC in the nonoverlap

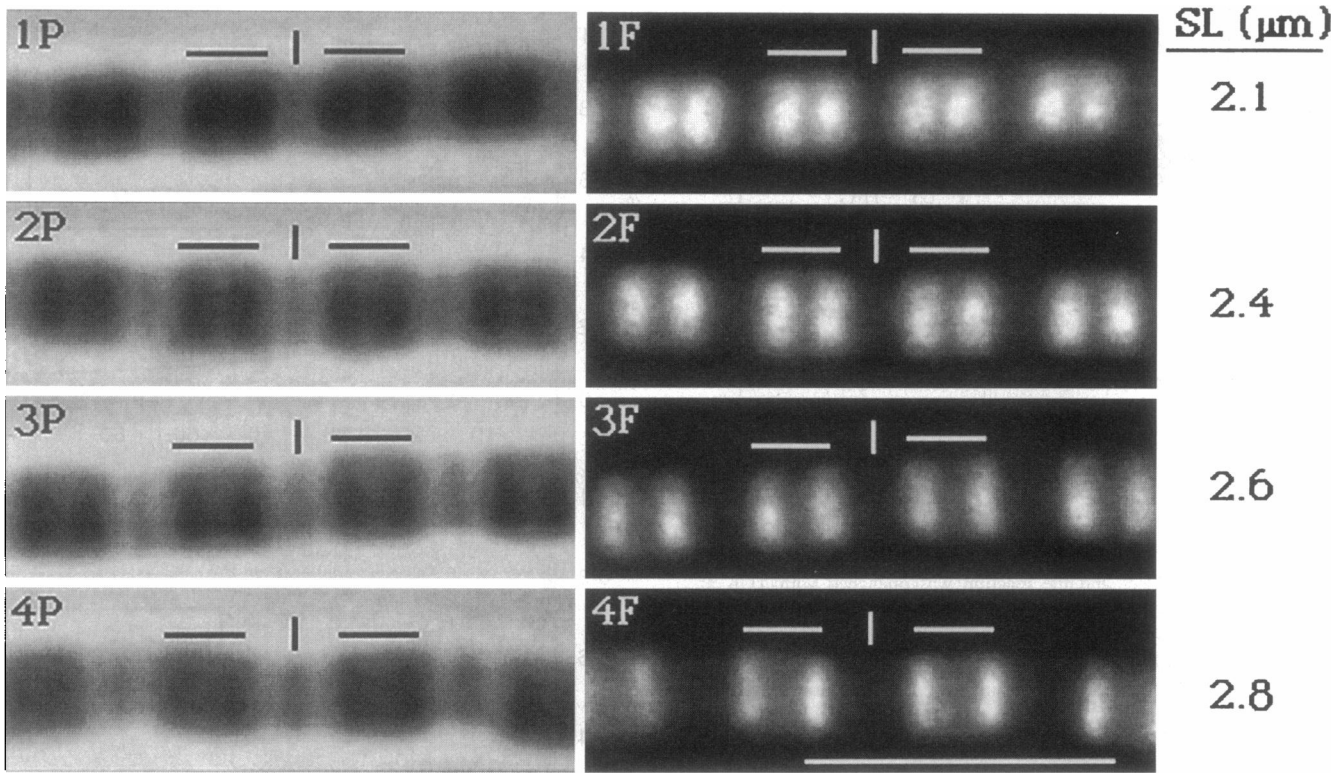

FIGURE 4 Fluorescein TnC binds in the overlap region at pCa 9.0 in extracted myofibrils. TnC-extracted myofibrils (12% relative calcium ATPase) were treated with 200 nM F5M TnC at pCa 9.0, as described in Materials and Methods. Both phase-contrast (P) and fluorescein (F) images are shown. Sarcomere lengths were 2.1, 2.4, 2.6, and 2.8 μm for myofibrils 1, 2, 3, and 4, respectively. The fluorescence pattern was sarcomere-length dependent, with most of the fluorescence in the actin-myosin overlap region. Similar results were obtained at higher levels of F5M TnC. Vertical lines denote Z-lines, short horizontal bars designate the A-band, and the scale bar is 5 μm.

region of the A-band, which is most obvious in Fig. 4, *4F*. These results suggest that either TnC is preferentially extracted from the overlap region or that the binding of F5M TnC is influenced by the cross-bridge bound to the thin filament.

To test the hypothesis that cross-bridge binding of the thin filament influences F5M TnC binding, highly extracted myofibrils were treated with 200 nM F5M TnC under conditions that resulted in different degrees of saturation of the thin filament with myosin heads. Fig. 5 shows a montage of myofibrils treated with F5M TnC under relaxing conditions (*1P* and *1F*), rigor (*2P* and *2F*), and rigor plus extrinsic myosin heads (*3P* and *3F*). Under relaxing conditions, there was little F5M TnC bound, and the location of the fluorescence is difficult to discern because of the noise in the image. Under rigor conditions, the fluorescence was localized to the actomyosin overlap region, as seen in Fig. 4. When additional extrinsic heads were added in the form of S1, the fluorescence was located along the entire length of the thin filament, except at the Z-line (Fig. 5, *3F*). The level of S1 was at or near saturating levels with respect to available actin sites (Swartz et al., 1990). These results strongly suggest that the binding of F5M TnC at pCa 9.0 to the TnC-denuded Tn complexes along the thin filament is influenced by strong myosin head binding to the thin filament instead of being due to preferential extraction of TnC from the overlap region.

To further investigate the characteristics of F5M TnC binding, the effects of calcium and the concentration of F5M TnC on the location of F5M TnC binding were measured. The concentration of TnC-binding sites can be estimated from the concentration of myofibrillar protein and the measured level of troponin per unit myofibrillar protein, as determined by Yates and Greaser (1983). For the concentration of myofibrillar protein used (0.25 mg/ml), the concentration of TnC sites was 180 nM. Assuming that 12% relative calcium-dependent ATPase activity represents 12% residual TnC, the concentration of sites in highly extracted myofibrils was 158 nM. Our studies used 25–200 nM F5M TnC, giving ~15–125% of the concentration of sites. Experiments with unextracted myofibrils showed very little binding of F5M TnC at high calcium and high levels of F5M TnC, suggesting that there was very little nonspecific binding of F5M TnC and that there was little exchange of labeled TnC for native TnC (data not shown). Fig. 6 shows a side-by-side comparison of the influence of calcium on the location of F5M TnC binding and a top-to-bottom comparison of the influence of F5M TnC concentration on the location of F5M TnC binding. At 25 nM F5M TnC (Fig. 6, *1F* and *5F*), calcium had little influence on the location of

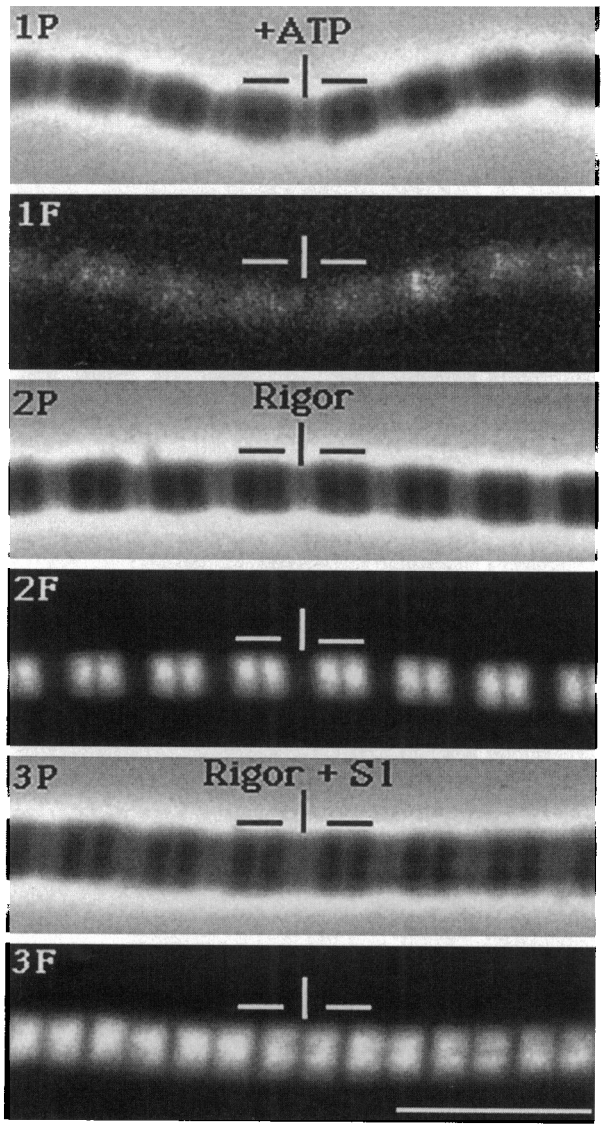

FIGURE 5 Fluorescein TnC binding is influenced by the cross-bridge status of the thin filament at low calcium. TnC-extracted myofibrils (5% relative calcium ATPase) were treated with 200 nM F5M TnC at pCa 9.0 under relaxing conditions (+ ATP, 1), rigor (2), or rigor + 1 μM S1 (3). Fluorescein TnC bound to the thin filament only in regions that contained bound myosin heads. Letters, vertical and horizontal lines, and the scale bar are as in Fig. 4.

TnC binding; it was localized in the overlap region, as shown for low calcium alone. Comparison of the calcium effect at 200 nM F5M TnC shows that there was an influence of calcium on the location of F5M TnC binding. At low calcium (Fig. 6, *4F*), it was localized to the overlap region, whereas at high calcium it was greatest in the overlap region, but significant binding was also observed in the nonoverlap region of the thin filament (Fig. 6, *8F*). The level of F5M TnC had little influence on the location of TnC binding at low calcium (compare Fig. 6, *1F–4F*), and even up to 1 mM F5M TnC similar patterns were observed (data not shown). The concentration of F5M TnC had an influence on the location of F5M TnC binding at high calcium in that there was a relative increase in the amount of fluorescence in the nonoverlap region as F5M TnC concentration was increased (compare Fig. 6, *5F–8F*). These images suggest that the concentration of F5M TnC influences the location of F5M TnC binding, but only at high calcium.

To compare the effects of both calcium and cross-bridge status of the thin filament, a montage of highly extracted myofibrils treated with 200 nM F5M at either low or high calcium and with or without S1 was made. Fig. 7 shows that, although calcium had an influence on the location of F5M TnC binding without added S1 (compare Fig. 7, *1F* and *3F*), it had little influence on the location of binding when saturating levels of S1 were present (compare Fig. 7, *2F* and *4F*). These latter images show that the fluorescence was along the length of the thin filament, with a slight difference in the intensity between the overlap and nonoverlap region (discussed below).

The above images suggest that the binding of F5M TnC is enhanced by both calcium and rigor heads. Thus, to determine the location of TnC extraction from rigor myofibrils, differentially extracted myofibrils were incubated with 200 nM F5M TnC at pCa 4.0 and with saturating levels of myosin heads to make the thin filament uniform along its entire length in terms of cross-bridge status. Fig. 8 shows myofibrils with 48 (*1P* and *1F*), 23 (*2P* and *2F*), and 12% (*3P* and *3F*) relative ATPase activity. At the lowest level of TnC extraction (*1F*), the F5M TnC bound predominantly in the nonoverlap region (I-band), with a lesser amount in the overlap region. With greater extraction (Fig. 8, *2F*), the difference between the nonoverlap and overlap regions was not as great, and at higher levels of extraction (*3F*), the difference between overlap and nonoverlap fluorescence intensities is barely observed. These images suggest that the extraction of TnC is not random, but rather occurs preferentially in the nonoverlap region and then in the overlap region.

The images were processed to emphasize the distribution of F5M TnC in extracted myofibrils under a variety of conditions. They do not readily yield information on the amount of F5M TnC bound. For this, we analyzed the fluorescence intensity from the 12-bit image data and compared the approximate amount of F5M TnC bound under different conditions and the relative, predominant location of the bound F5M TnC. Fig. 9 shows the sarcomeric intensity of F5M TnC at high and low calcium and with and without S1 as a function of F5M TnC concentration. The most striking feature observed was that calcium increased the sarcomeric fluorescence intensity 5- to 10-fold over the concentration range of F5M TnC studied. The addition of S1 had little influence on the sarcomeric intensity at high calcium, whereas it increased the sarcomeric intensity by about twofold at low calcium. Fitting the data to a hyperbolic equation gave estimates of the maximum sarcomeric intensity and the approximate dissociation constant for F5M TnC. Interestingly, the fitted dissociation constants ranged from 35 to 60 nM for the different conditions, whereas the maximum intensity was lowest for low calcium without S1

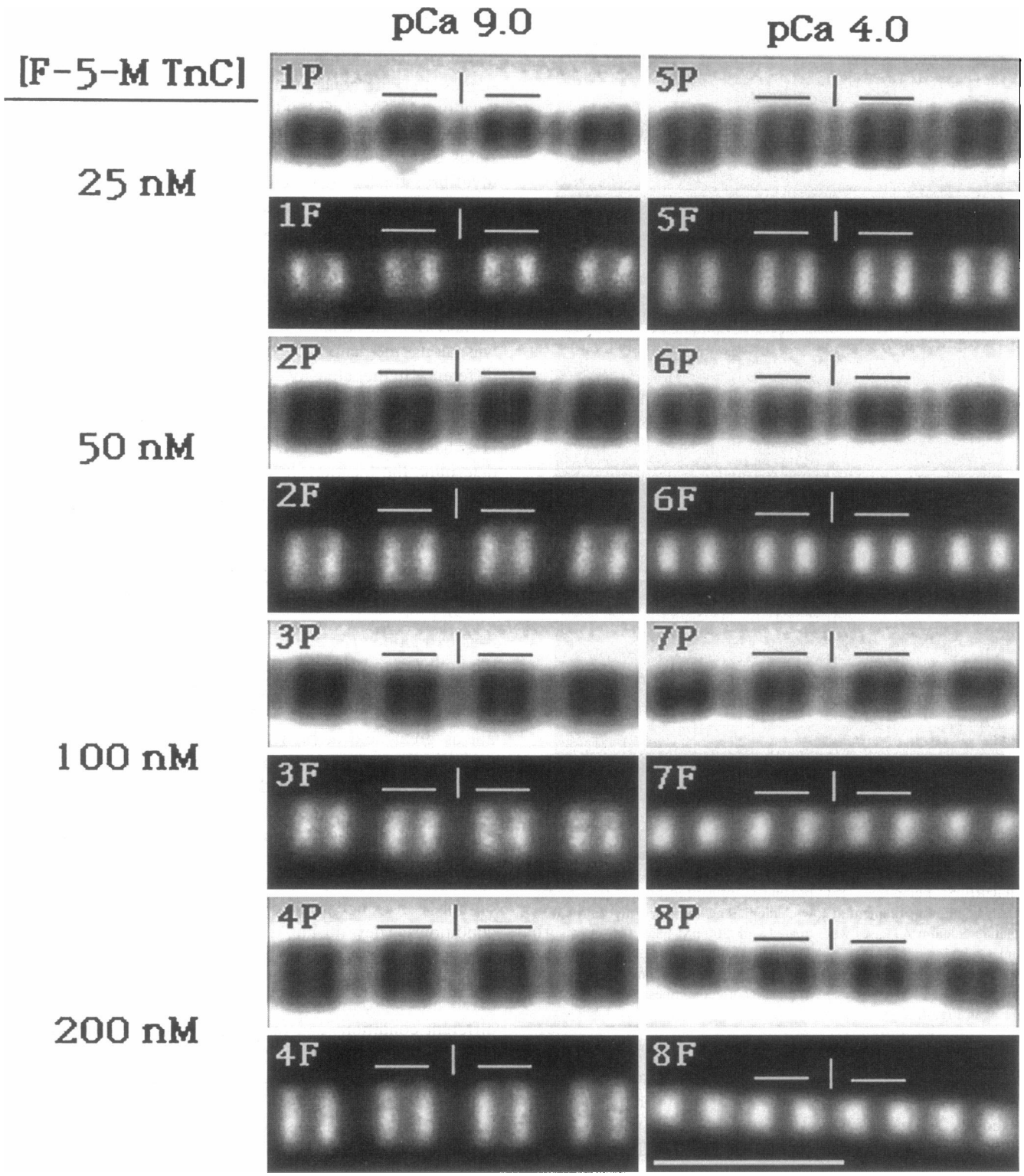

FIGURE 6 The location of F5M TnC binding is influenced by both F5M TnC concentration and calcium level. TnC-extracted myofibrils (12% relative calcium ATPase) were incubated with different levels of F5M TnC at either pCa 9.0 (1–4) or pCa 4.0 (5–8) and processed as described in Materials and Methods. The pattern at pCa 9.0 was insensitive to F5M TnC concentration, whereas at pCa 4.0 increasing amounts of fluorescence were found in the I-band as F5M TnC increased. Letters, vertical and horizontal lines, and the scale bar are as in Fig. 4.

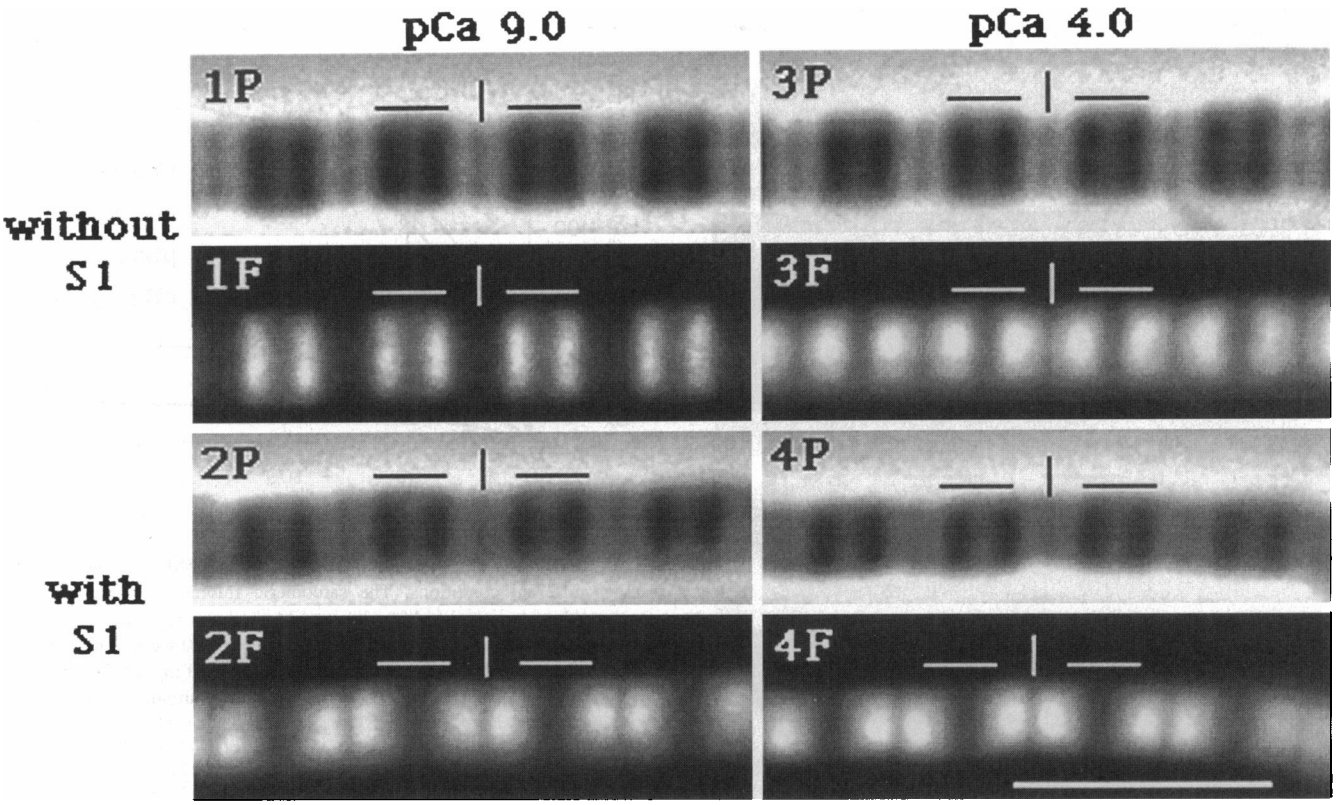

FIGURE 7 Comparison of F5M TnC binding at pCa 9.0 and 4.0 without and with extrinsic myosin heads. TnC-extracted myofibrils (12% relative calcium ATPase) were treated with 200 nM F5M TnC at pCa 9.0 (1 and 2) or pCa 4.0 (3 and 4) without (1 and 3) or with (2 and 4) 1 μM S1 and processed as described in Material and Methods. Treatment of myofibrils without S1 at pCa 9.0 showed that the fluorescence was localized to the overlap region (1F), whereas with S1 it was more uniformly distributed along the entire length of the thin filament (2F). At pCa 4.0 without S1, the fluorescence was highest in the overlap region with lesser amounts in the nonoverlap region, whereas with S1, the fluorescence was distributed more evenly. Letters, vertical and horizontal lines, and the scale bar are as in Fig. 4.

and highest for high calcium without S1. This analysis suggests that calcium and heads influence the number of binding sites but not their affinity.

The sarcomeric intensity data do not address the location of the fluorescence within the sarcomere. To measure this, the ratio of the fluorescence intensity in the nonoverlap region relative to that in the overlap region was determined. This value will be low if most of the F5M TnC is bound in the overlap region and will be higher if more is bound in the nonoverlap region. Fig. 10 shows the intensity ratio as a function of F5M TnC concentration for myofibrils at pCa 4.0 and pCa 9.0 without S1. The data show that at low calcium, there is no change in the relative distribution of F5M TnC as a function of F5M TnC level, whereas at high calcium, increases in F5M TnC concentration result in more being bound in the nonoverlap region. This graphical representation of the change in distribution at high calcium was observed in the images as a spreading of the fluorescence from the overlap region into the nonoverlap region (see Fig. 6, *5F–8F*).

Table 1 summarizes the data for the properties of partially extracted myofibrils shown in Fig. 8. At 43% relative pCa 4.0 ATPase activity, the intensity ratio was 1.78, suggesting that there was more F5M TnC bound in the nonoverlap region than the overlap region, corresponding to a greater amount of native TnC being extracted from the nonoverlap region than the overlap region. At 12% relative pCa 4.0 ATPase activity, a level where most of the native TnC was extracted, the ratio was 1.3, suggesting that the difference in the amount of native TnC in the nonoverlap and overlap regions was much less. The sarcomeric intensity data show that with lowered relative pCa 4.0 ATPase activity, there was an increase in the sarcomeric intensity, as would be expected if more native TnC were removed.

DISCUSSION

The studies described above were designed to determine the structural location of TnC extraction at the myofibril level by using a fluorescent conjugate of TnC in conjuction with high-resolution fluorescence microscopy. An advantage of this approach is that it also allows for characterization of the interaction of TnC with the native thin filament in the intact myofilament lattice, which has not been done before. In fact, our initial experiments forced us to characterize the interaction of F5M TnC with highly extracted myofibrils, so that the conditions that led to the greatest incorporation of

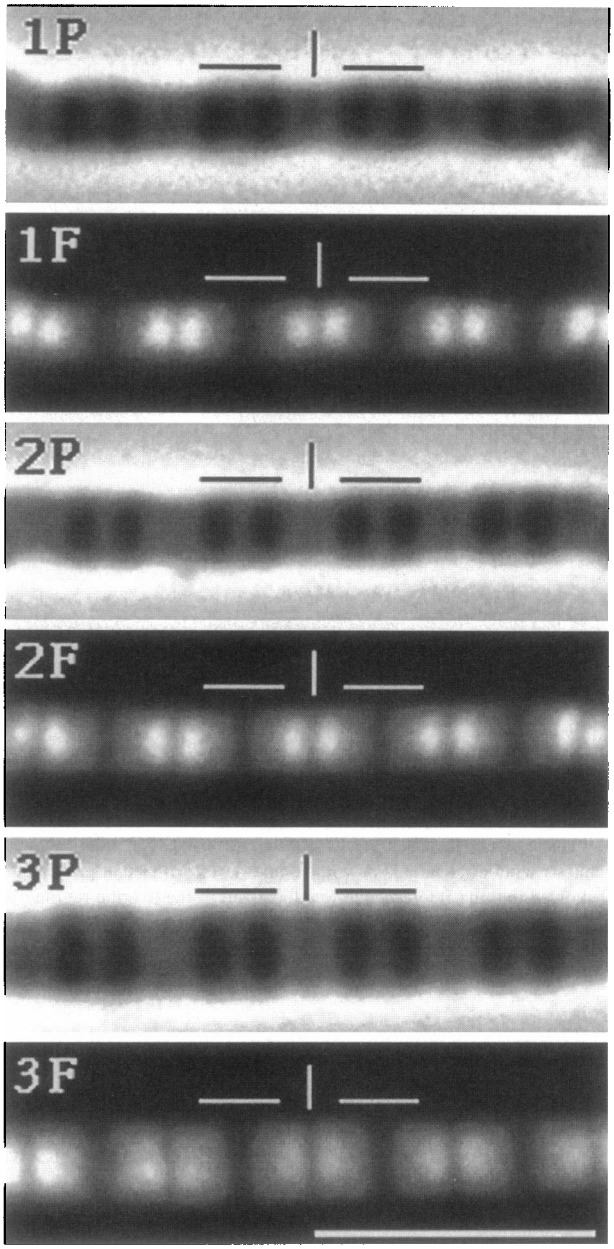

FIGURE 8 Troponin C was not randomly extracted along the length of the thin filament. Myofibrils with different levels of TnC extraction were treated with 200 nM F5M TnC at pCa 4.0 with 1 μM S1 and processed as described in Materials and Methods. At low levels of extraction (higher relative ATPase), most of the F5M TnC was localized to the nonoverlap (1F) region, whereas at high levels of extraction, the F5M TnC was more evenly distributed in both the nonoverlap and overlap regions. Letters, vertical and horizontal lines, and the scale bar are as in Fig. 4.

F5M TnC could be applied to partially extracted myofibrils. This characterization helped in explaining why extraction is not random along the thin filaments of myofibrils.

Properties of F5M TnC interaction with native binding sites

Our initial experiments characterized the binding of F5M TnC to highly extracted myofibrils at low calcium. Those

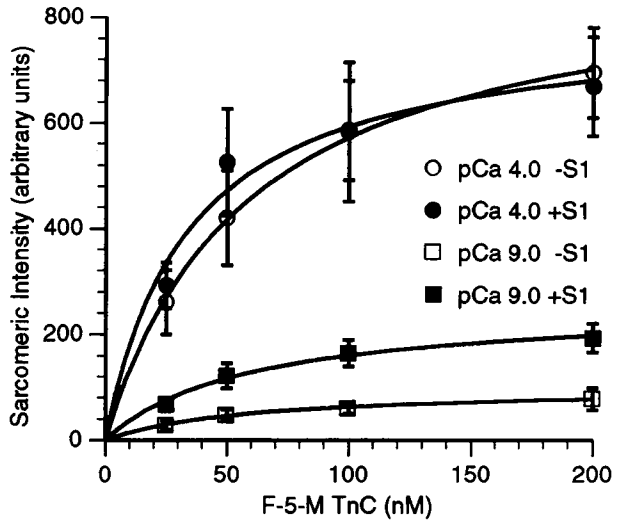

FIGURE 9 Sarcomeric intensity as a function of F5M TnC concentration under different conditions. The sarcomeric fluorescence intensity was measured as described in Materials and Methods for myofibrils incubated at pCa 4.0 without (○) or with (●) S1 and at pCa 9.0 without (□) or with (■) S1. Data points represent the mean ± SD of 20 myofibrils. The data were fit with a hyperbolic relation of the form sarcomeric intensity = maximum intensity * [F5M TnC]/(K_d + [F5M TnC]). The fitted values for maximum intensity/K_d were 800/34 nM (pCa 4.0, +S1), 914/59 nM (pCa 4.0, −S1), 258/60 nM (pCa 9.0, +S1), and 102/60 nM (pCa 9.0, −S1).

studies demonstrated that the F5M TnC bound in the overlap region, and the pattern was dependent upon sarcomere length (Fig. 4). There was some binding of F5M TnC in the nonoverlap region of the A-band, suggesting that some of the F5M TnC bound to myosin (see Fig. 4, *4F*). This was observed at the lowest levels of F5M TnC tested (25 nM; see Fig. 6, *1F*), suggesting that it was specific binding, and it was not observed in unextracted myofibrils. The binding in this region of the sarcomere may have been F5M TnC binding to the myosin head, inasmuch as others have observed low-level light-chain 2 extraction with TnC extraction (Zot and Potter, 1982). The sarcomere-length-dependent binding suggests that the F5M TnC had a preference for binding in the actomyosin overlap region of the A-band and thus that strong binding of the myosin head to actin favors TnC binding. To further test this idea, highly extracted myofibrils were treated F5M TnC under conditions of relaxing (no strong binding), rigor (strong binding in the overlap region), and rigor plus saturating S1 (strong binding in both overlap and nonoverlap regions). These images (Fig. 5) support the idea that the binding of TnC is favored by strong binding of the myosin head to actin at low calcium.

The location of F5M TnC binding was also influenced by calcium and the concentration of F5M TnC in highly extracted myofibrils. At low calcium, F5M TnC bound primarily in the overlap region, and the pattern was not influenced by F5M TnC concentration over the range tested. At high calcium, the pattern of F5M TnC was influenced by F5M TnC concentration, with the location of binding spreading from the overlap region into the nonoverlap re-

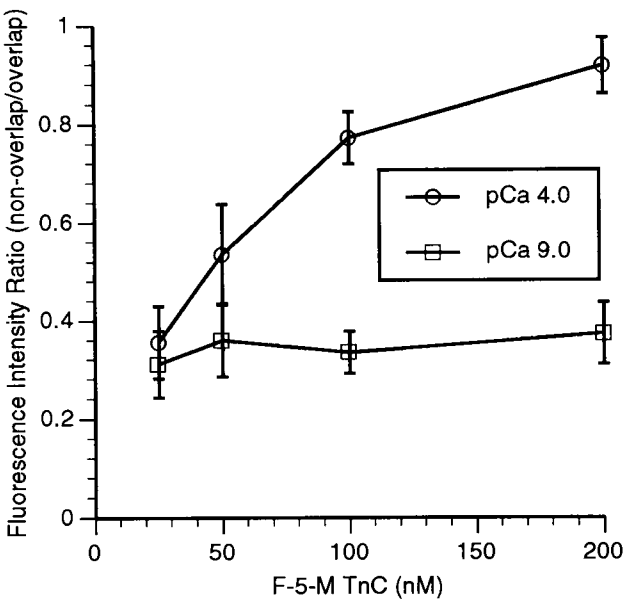

FIGURE 10 Fluorescence intensity ratio of nonoverlap to overlap regions as a function of F5M TnC concentration at high and low calcium. Fluorescence intensity ratios were measured as described in Material and Methods for myofibrils incubated with F5M TnC at pCa 4.0 (○) or pCa 9.0 (□). The data points represent the mean ± SD of 20 myofibrils. There was no change in the ratio at pCa 9.0, whereas there was an increase at pCa 4.0.

gion with increases in F5M TnC concentration (compare Fig. 6, *5F–8F*). The characteristics of the location of F5M TnC binding as a function of F5M TnC concentration at low and high calcium are shown in Fig. 10 as the intensity ratio of the nonoverlap to overlap regions. The graph demonstrates that there was no change in the ratio at low calcium. However, there was an increase in the ratio with increased F5M TnC at high calcium, suggesting that the binding propagated from the overlap region into the nonoverlap region. This propagation supports the idea of cooperative interactions between nearest-neighbor functional units along the thin filament that are likely propagated by tropomyosin (Pan et al., 1989; Geeves and Lehrer, 1994).

At high calcium, the addition of saturating levels of S1 influenced the pattern of F5M TnC binding such that there was more bound in the nonoverlap region than the overlap region (Fig. 7, compare *3F* and *4F*). The intensity ratio for these highly extracted myofibrils was 1.3 (Table 1), compared to 0.92 for myofibrils without S1 at the same level of F5M TnC. Thus the binding of TnC was influenced by strongly binding heads, even at high calcium levels. These observations suggest that the strong binding of myosin heads to actin influences the affinity of the binding site for TnC along the thin filament, even under high calcium conditions.

Quantitative analysis of the fluorescence intensity per sarcomere was done to determine the extent of the influence of calcium and S1 upon the amount of F5M TnC bound. This analysis assumes that the spectral properties of the fluorescein attached to TnC were not influenced by calcium

TABLE 1 Characteristics of partially TnC-extracted myofibrils

No. of extractions	Relative ATPase	Intensity ratio	Sarcomeric intensity
1	43%	1.78 ± 0.085	127 ± 43
2	23%	1.48 ± 0.066	209 ± 40
8	12%	1.30 ± 0.047	248 ± 41

*Mean ± SD.

or rigor head attachment to the thin filament. Some support for this assumption is that after fixation, all myofibrils were imaged in the same, low-calcium, mounting medium and that at low calcium, rigor heads increased the amount bound, whereas at high calcium, it had little influence on the amount bound. Fig. 9 shows the relationship between F5M TnC concentration and sarcomeric fluorescence intensity. The most striking feature of this figure is that calcium increased the amount bound, whereas it had little influence on the concentration for half-maximum binding. These data can be interpreted as calcium increasing the number of TnC binding sites. This can be explained by calcium increasing the affinity of TnC for TnI, likely by saturation of the regulatory sites of TnC with calcium. The addition of S1 had an influence on the amount bound at low calcium, but did not influence the concentration of F5M TnC for half-maximum binding. This fits with the observation that, without S1, the F5M TnC binding was restricted to the overlap region, whereas with S1, it was in both the overlap and nonoverlap regions at low calcium. These observations together fit with the idea that calcium influenced the ligand (F5M TnC), whereas strong binding heads influenced the binding site (i.e., TnI and TnT).

An effect of calcium on the interaction of TnI with TnC is well documented, in that calcium increases the binding of TnC to TnI (Ingraham and Swenson, 1984; Leavis et al., 1984; Wang and Cheung, 1985; Cheung et al., 1987) and our data from the solid-phase assay support this conclusion. The influence of strongly binding heads on the troponin complex is less direct but suggests that this binding has an influence on troponin. Rigor and force-generating heads have been shown to increase the affinity of TnC for calcium in myofibrils and skinned muscle preparations (Bremel and Weber, 1972; Fuchs and Fox, 1982; Hofmann and Fuchs, 1987; Cantino et al., 1993). Strongly binding heads have been shown to influence the conformation of TnC, as measured by conformationally sensitive fluorescent probes covalently attached to these subunits (Guth and Potter, 1987; Hannon et al., 1992). Thus our structural data on the characteristics of TnC binding to the thin filament within the myofibril are supported by other studies on these interactions, using both intact systems and isolated proteins.

Properties of TnC extraction

When partially extracted myofibrils were characterized in terms of the location of F5M TnC binding, we observed that

more F5M TnC was bound in the nonoverlap region than in the overlap region. With increases in extraction, this difference became less. This is shown structurally in Fig. 7 and quantitatively in Table 1. Higher levels of extraction were associated with a decrease in the intensity ratio of the nonoverlap/overlap intensity and with an increase in the total amount of F5M TnC bound (sarcomeric intensity). These structural and quantitative data show that the extraction of TnC from the native thin filament is not random but favored in the nonoverlap region. The characteristics of F5M TnC binding can be used to explain the nature of TnC extraction from rigor myofibrils. The binding of TnC to TnI is favored under conditions with high levels of calcium and/or magnesium. The inclusion of EDTA in the extraction solution will chelate both calcium and magnesium, thus weakening the interaction between TnC and TnI. The observation that rigor heads enhance the binding of F5M TnC at low calcium results in nonuniform extraction along the length of the thin filament. In the nonoverlap region, there are no rigor heads attached, so that the interaction between TnC and TnI is less favored and thus the TnC dissociates from this region more readily. This may explain the observations of Brandt et al. (1987) of the fast extracting component that was not associated with loss of force. The TnC in the nonoverlap region was not likely associated with force and was likely of lower affinity and thus had a faster off rate than the TnC in the overlap region.

Properties of TnC extraction and relation to physiological studies

First, our studies demonstrate that extraction of TnC from the thin filament is not random, but occurs preferentially in regions of the thin filament devoid of strongly binding myosin heads. This complicates interpretations of cooperative mechanisms of thin filament activation based upon force data obtained from partially extracted fibers. Our observations do not support the model of Brandt et al. (1987), which was based upon random extraction of TnC along the entire thin filament. Second, studies using TnC extraction, in which the native TnC is replaced by a mutant or labeled TnC, are complicated by the residual native TnC in the sarcomere. This residual TnC likely remains in the overlap region, whereas there will be very little in the nonoverlap region. Thus reconstitution with a different TnC will result in a nonuniform distribution of the different form of TnC along the thin filament. The best approach is to highly extract the myofibrils or fibers so that there is less than 10% of residual ATPase activity or force remaining. This should result in a more uniform distribution of TnC reassembled along the length of the thin filament. Third, our data on the location of TnC extraction from myofibrils do not address the uniformity of extraction across the diameter of a skinned fiber. Yates et al. (1993) concluded that fiber diameter did not affect TnC extraction uniformity, based upon quantitative SDS-PAGE and force data, but structural data from confocal microscopy of extracted fibers reconstituted with a fluorescent TnC will be needed to more accurately address the issue of uniformity across the fiber diameter. Last, our data demonstrate that strong binding of the myosin head to actin in the native thin filament enhances the interaction of TnC with the TnI-TnT; this is consistent with other biochemical data suggesting reciprocal coupling of myosin head binding and thin filament activation.

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