Effect of Removing the Amino-Terminal Hexapeptide of Tropomyosin on the Properties of the Thin Filament†

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ABSTRACT: The role of the amino-terminal region of α-striated muscle tropomyosin has been analyzed by reconstituting thin filaments with a version of the protein lacking the first six amino acids. While Omp T-digested tropomyosin (residues 7−284) does not bind significantly to F-actin at micromolar concentrations, an interaction is induced by skeletal troponin. At a moderate ionic strength (50 mM KCl), the apparent K_d values are 0.26 μM (with EGTA) and 1.6 μM (with Ca^{2+}). At higher neutral salt (140 mM KCl), reconstitution is observed in the micromolar range only at high pCa (K_d = 1.3 μM). However, when chloride is replaced by acetate, the binding isotherms reach saturation under both extremes of Ca^{2+} [apparent K_d values of 0.32 μM (with EGTA) and 2.6 μM (with Ca^{2+})]. The induction of binding of truncated tropomyosin to F-actin by troponin is attributable, in part, to troponin-I, but whereas the amino-terminal fragment of troponin-T (N-Tn-T, residues 1−158) enhances the effect of troponin-I in the case of other tropomyosin products specifically, unacetylated tropomyosin (residues 1−284), and carboxypeptidase-digested tropomyosin (residues 1−273) [Heeley, D. H., et al. (1987) *J. Biol. Chem. 262*, 9971−9978], it is ineffective with regard to Omp T-digested tropomyosin, suggesting that cleavage has disrupted a binding site for this section of troponin-T. Thin filaments (with Ca^{2+}) containing Omp T-digested tropomyosin activate the steady-state myosin-MgATPase to a greater extent than the integral system, consistent with the interaction between N-Tn-T and the amino-terminal region of tropomyosin having a regulatory function. At high pCa, the truncated system exhibits a less cooperative interaction with myosin-S1-ADP but the affinity for the ligand is stronger. In context with the current methodologies, the consequences of shortening tropomyosin at one end as opposed to the other are the reverse of each other.

Tropomyosin is a coiled coil protein consisting of two α-helical chains (*1*). In striated muscle, it exists in association with F-actin (*2*) and troponin (*3*) as well as in a self-polymerized form (*4, 5*). The latter interaction, which is proposed to arise by insertion of the first 10 or so amino acids into a "jaw" created by opening of the carboxyl termini (*5*) and shows up in crystal lattices as a zone of increased thickness (*6*), allows tropomyosin to span the length of the thin filament. Within this complex, troponin-T is positioned along much of the carboxyl-terminal half of tropomyosin via its tail-like, amino-terminal section (*7*−*12*).

The end regions of tropomyosin have been investigated biochemically by group modification (*11, 13*−*17*) and subtraction (*9, 10, 18*−*20*) of the primary structure. Exolytic digestion results in the removal of 11 amino acids from the carboxyl-terminal end and significantly disrupts polymerization and interaction with F-actin (*20*). However, the shortened protein (residues 1−273) was successfully reconstituted into thin filaments at micromolar concentrations, and high and low pCa, in the presence of troponin (*9, 10*). By comparison, a genetically derived tropomyosin lacking the first nine amino acids did not reassemble into thin filaments at low pCa (*17, 18*).

A more recent approach, the endolytic digestion of muscle tropomyosin by bacterial outer membrane protease T (Omp T),[1] generates a product that is lacking the first six amino acids (*21*−*23*). As expected, the shortened protein, encompassing residues 7−284, exhibits weakened affinity for F-actin (*23*). Working with solutions of moderate or low ionic strength, containing either part-millimolar EGTA or Ca^{2+}, we observe full incorporation of Omp T-digested tropomyosin into thin filaments. This has permitted investigation of the roles of tropomyosin's amino-terminal region in thin filament structure and function.

Evidence that indicates that removal of the first six amino acids of tropomyosin disrupts the binding of the troponin-T tail (or N-Tn-T) is presented, inferring that within an intact system N-Tn-T interacts with surfaces in each half of tropomyosin. First, under conditions where N-Tn-T promotes the interaction of F-actin and tropomyosin products that possess the first six amino acids but otherwise have weak affinity for F-actin (*10*), it is ineffective in the case of Omp T-digested tropomyosin. This observation agrees with those from the crystallographic study (*24*) in which N-Tn-T was seen to reach beyond the overlap into the next tropomyosin in the chain, as well as those from NMR experiments in which a weak but specific interaction between N-Tn-T and

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[1] Abbreviations: N-Tn-T, amino-terminal chymotryptic fragment of troponin-T; Omp T, outer membrane protease T.

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tropomyosin fragments containing the first 10 amino acids was reported (*25*). Second, at high Ca^{2+} concentrations and in the presence of millimolar MgATP concentrations, thin filaments containing Omp T-digested tropomyosin activate myosin-S1 to a greater extent than control thin filaments, consistent with a change in N-Tn-T-based inhibition (*16*). Further, at low Ca^{2+} concentrations and in the absence of ATP, thin filaments composed of Omp T-digested tropomyosin display stronger affinity for myosin-S1-ADP than the control. Interestingly, the reverse is true on all of the above counts for tropomyosin digested with carboxypeptidase (*26, 27*). A preliminary version of this research has appeared (*28*).

EXPERIMENTAL PROCEDURES

Protein Preparation. Tropomyosin was isolated from the back muscle of New Zealand white rabbits (*29*), which comprises mostly the α-isoform and a small amount of the *β*-isoform (*30, 31*). Amino-terminally shortened tropomyosin (residues 7−284) was prepared as described previously (*23*). Specifically, we conducted large-scale digestion by harvesting a 3 L overnight culture of JM109, washing the cells in 0.1 M NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), and 50 mM phosphate (pH 7), and resuspending the cells in 300 mL of the same buffer with addition of 100 mg of rabbit skeletal muscle tropomyosin (∼10 mg/mL in the same buffer). The reaction mixture was incubated at 37 °C with gentle stirring for 4 h. The cells were removed by sedimentation, and the truncated tropomyosin-containing supernatant was passed sequentially over anion-exchange and hydroxyapatite chromatographic media. Removal of the amino-terminal hexapeptide was verified by a combination of electrophoresis, Edman-based sequencing, and mass spectrometry (*23*). Recombinant salmon fast skeletal muscle tropomyosin was prepared as described in ref *22* and also subjected to cleavage by Omp T. Actin (*32*), whole troponin (*33*), troponin-I (*10*), the amino-terminal chymotryptic fragment of troponin-T (N-Tn-T, residues 1−158) (*34*), and myosin subfragment-1 (S1) (*35*) were isolated from rabbit skeletal muscle as described previously. Protein enrichment was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*36*).

F-Actin Binding. Binding was investigated by sedimentation in an Airfuge (Beckman) at room temperature. Tropomyosin was labeled with iodo[1-^{14}C]acetamide (Amersham Biosciences). The reacted protein was dialyzed against the binding buffer until the counting rate of the bathing solution was background. Stock solutions of protein were made up by dialysis overnight against binding buffer and stored at 4 °C. Whole troponin was used within 1−2 days of the overnight dialysis. F-Actin was used within 1 week. The following extinction coefficients (ε_{280}, 1 mg/mL) were used after correction for scatter by subtraction of $1.5A_{320}$: tropomyosin, 0.25; troponin, 0.47; troponin-I, 0.4; and N-Tn-T, 0.143. The level of F-actin was determined by the relationship given in ref *37*. F-Actin, [^{14}C]tropomyosin, and, if present, troponin, troponin-I, or N-Tn-T were combined at final concentrations of 7, 0.25−9, and 2 μM. Proteins were gently mixed, incubated for 45 min at 4 °C, and then centrifuged for 30 min at 150000*g*. Two 10 μL aliquots of samples were removed before and after centrifugation for counting. The difference was attributed to the amount of tropomyosin bound. Correction for aggregated tropomyosin was made by sedimentation in the absence of other proteins.

Actomyosin MgATPase. Thin filaments were reconstituted separately with either Omp T-digested or native tropomyosin. Buffer consisted of 10 mM MOPS, 4.5 mM $MgCl_2$, 1 mM DTT (pH 7), and 0.5 mM EGTA or Ca^{2+}, with an ionic strength of 20 mM at 25 °C. Protein mixtures, containing a supplement of 2 mM $MgCl_2$, were incubated at 4 °C for 30 min and heated to 25 °C, and then the reaction was initiated by addition of 2 mM ATP. The amount of inorganic phosphate was determined colorimetrically according to ref *38*. Five time points were taken to determine the rate of reaction.

Myosin-S1 Binding. Thin filaments, assembled with either truncated or native tropomyosin, were mixed with varying concentrations of myosin-S1A1 in a buffer composed of 5.5 mM $MgCl_2$, 50 mM KCl, 10 mM imidazole, 3 mM MgADP, 0.5 mM EGTA or Ca^{2+}, and 1 mM DTT (pH 7). Included in the mixture was 20 μM myokinase inhibitor (Ap5A, Sigma). The samples were incubated at 4 °C for 1 h and then centrifuged in the Airfuge (Beckman) for 30 min at 150000*g*. The amount of unbound S1 was determined by an NH_4/EDTA ATPase assay at 25 °C. Since the zero time samples from this assay showed no color development, the binding mixtures are assumed to be free of ATP.

RESULTS

Effect of Whole Troponin and Subcomponents Tn-I and N-Tn-T (residues 1−158) on the Binding of Omp T-Digested Tropomyosin (residues 7−284) to Actin. The binding of Omp T-digested tropomyosin to F-actin in the presence of skeletal troponin was assessed by a sedimentation assay using radiolabeled protein. The digested tropomyosin was material that had been rigorously validated by a combination of electrophoresis, Edman-based sequencing, and mass analysis and was confirmed as encompassing residues 7−284 (*23*). The source of the tropomyosin, unless otherwise stated, is bulk rabbit skeletal muscle, which comprises mostly the α-isoform (*30, 31*). The inset of Figure 1A shows that the product of Omp T digestion electrophoretically migrates slightly ahead of the parent, consistent with the removal of a small peptide.

Reconstitution at an ionic strength of 70 mM [50 mM KCl, 5.5 mM $MgCl_2$, and 10 mM MOPS (pH 7)] is described. The Ca^{2+} concentration was varied by including, in the solution described above, either 0.5 mM $CaCl_2$ (pCa < 5) or 0.5 mM EGTA (pCa > 7). Binding isotherms (Figure 1A) were constructed by increasing the concentration of Omp T-digested tropomyosin from 0.25 to 9 μM while maintaining the other components (F-actin and skeletal troponin) at constant concentrations. Under these conditions, Omp T-digested tropomyosin does not cosediment with F-actin to any measurable extent in the absence of troponin (data not shown), but when troponin is present, the binding curves ascend to a plateau which coincides, within experimental error, with one tropomyosin per seven actin monomers (Figure 1A). The ability of troponin to induce the binding of Omp T-digested tropomyosin to F-actin is Ca^{2+} sensitive, being approximately 6-fold stronger at higher pCa than lower (Figure 1A). The apparent K_d values for the binding of Omp T-digested tropomyosin to F-actin are 0.26 ± 0.03 μM (two

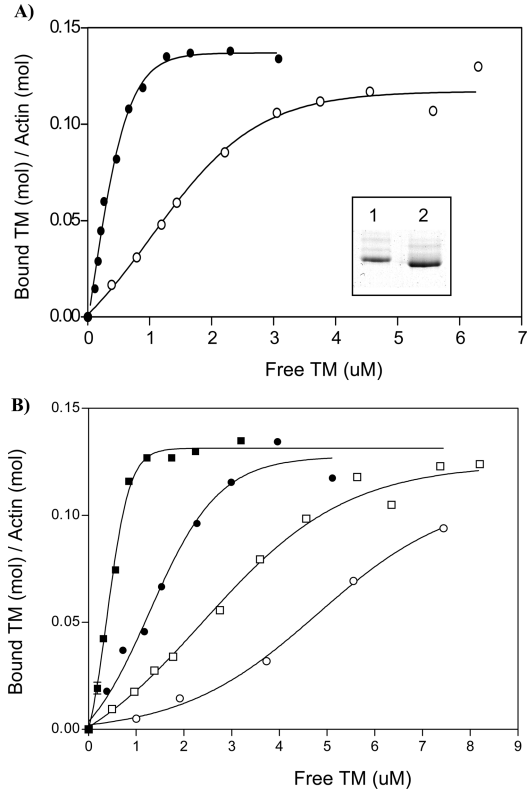

FIGURE 1: Induction of binding of Omp T-digested rabbit striated muscle α-tropomyosin to F-actin by troponin (with EGTA and Ca^{2+}). Actin and troponin concentrations were kept constant at 7 and 2 μM, respectively, while the tropomyosin (residues 7−284) concentration was varied; 100% sedimentation of F-actin was assumed. A small amount of aggregated Omp T-cleaved tropomyosin (<5% of the total) was corrected for by sedimentation in the absence of other proteins. When this is taken into account, there is no detectable sedimentation of the shortened tropomyosin in the presence of F-actin but without troponin. Curves were fit using the equation $Y = B_{max}/[1 + 10^{(\log K_d - \log X)n}]$ (GraphPad Prism). (A) The buffer consisted of 10 mM MOPS, 50 mM KCl, 5.5 mM $MgCl_2$, 1 mM DTT (pH 7) with an ionic strength of 70 mM, and 0.5 mM EGTA (●) or Ca^{2+} (○) at 20 °C. The K_d values, 0.24 μM (EGTA) and 1.6 μM (Ca^{2+}), are close to the values cited in Results which represent the average of three independent determinations. The inset is a Coomassie Brilliant Blue R-250-stained SDS−polyacrylamide gel window of rabbit striated α-tropomyosin: lane 1, native; lane 2, Omp T-digested. (B) The buffer consisted of 10 mM MOPS, 140 mM KCl (circles) or 140 mM KAc (squares), 5.5 mM $MgCl_2$, 1 mM DTT (pH 7), and 0.5 mM EGTA (filled symbols) or Ca^{2+} (empty symbols) at 20 °C. K_d values were 0.32 μM (140 mM KAc with EGTA), 1.4 μM (140 mM KCl with EGTA), and 3.2 μM (140 mM KAc with Ca^{2+}). These values compare favorably with those cited in Results which are the average of three independent determinations. Note that the acetate-containing buffer was set to neutrality only after dissolution of acetate.

independent experiments, *n*) and 1.65 ± 0.28 μM ($n = 3$) (Table 1). A final point is that the isotherms exhibit some cooperativity: Hill coefficients of 1.58 (EGTA) and 1.15 (added Ca^{2+}).

To reconcile these results with previously published work (*17, 18*), the experiments were repeated in a higher-ionic strength neutral salt buffer [140 mM KCl, 5.5 mM $MgCl_2$, and 10 mM MOPS (pH 7)] (Figure 1B). This buffer is compatible in ionic strength, 163 μM, to the one used previously with chicken and rat skeletal muscle tropomyosin lacking the first nine residues (*17, 18*). Increasing the ionic strength in this manner weakens the induction of binding by troponin (Figure 1B). At high pCa, the apparent binding constant ($K_d = 1.3 \pm 0.14$ μM; $n = 4$) is reduced ~5-fold compared to what was measured at an ionic strength of 70 mM (Figure 1A) and compares favorably with what was reported for the slightly shorter tropomyosin product (*17*). Also in agreement with the previous work, saturation was not attained at low pCa within the protein concentration range that was used (Figure 1B).

The effect of ionic strength is not straightforward, however. Replacing 140 mM neutral salt with an equivalent amount of potassium acetate produces a leftward shift in both isotherms (Figure 2). With added EGTA, the apparent K_d is 0.32 ± 0.00 μM ($n = 2$), 4 times stronger than that observed with 140 mM KCl. With added Ca^{2+}, the apparent binding constant is 2.6 ± 0.6 μM ($n = 3$) (Table 1). The authors cannot offer a complete explanation for the observed anion dependence. It could be that acetate is less disruptive than chloride with respect to protein−protein interactions, because of its larger size and resonance charge stabilization. Whatever the reason, the concentration and nature of the salt contained within the low-Ca^{2+} concentration binding buffer appear to have a greater influence than the size of the reduction of the amino-terminal region, unless rabbit striated α-tropomyosin is inequivalent (in context with the current methodologies) to α-tropomyosins from other animal sources.

Experiments involving subcomponents of troponin (Figure 2) are described next. Proteins were dispersed in 70 mM ionic strength buffer as per Figure 1A. Troponin-I was added alone and in combination with the soluble chymotryptic fragment, N-Tn-T, which represents the tail portion of troponin-T. It is evident that troponin-I, by itself, can restore interaction of Omp T-digested tropomyosin with F-actin ($K_d = 1.0 \pm 0.00$ μM; $n = 2$) (Figure 2A). However, when the N-Tn-T peptide is included in the assay mixture (i.e., F-actin, tropomyosin residues 7−284, troponin-I, and N-Tn-T), the isotherm is virtually superimposable with the case where the fragment is absent. Interestingly, N-Tn-T was consistently shown to accentuate the effect of troponin-I in an identical assay involving carboxyl-terminally shortened tropomyosin (*10*). To build on this point, experiments were performed with an unacetylated, full-length tropomyosin (residues 1−284) which also exhibits insignificant F-actin binding in the micromolar range. In this instance, N-Tn-T is observed to accentuate the effect of troponin-I (Figure 2B), but no such enhancement occurs with the same recombinant tropomyosin which had been cleaved with Omp T (Figure 2C). Thus, the question of whether N-Tn-T has an effect in the induction assay depends upon the presence or absence of the first six residues of tropomyosin.

Regulatory Properties of Thin Filaments Containing Omp T-Digested Tropomyosin. The ability to reconstitute thin filaments allowed for the effects of the amino-terminal truncation of tropomyosin on regulation of actomyosin-S1 MgATPase to be studied. Assays were performed in a low-salt buffer (ionic strength, ~20 mM). Under these conditions, the expectation was that binding would be no weaker than what was observed at an ionic strength of 70 mM (Figure 1A). This was verified by sedimentation (Figure 3, inset). The apparent K_d values are 0.19 ± 0.01 μM (with EGTA; $n = 2$) and 0.83 ± 0.11 μM (with Ca^{2+}; $n = 3$), twice as strong as what is observed at an ionic strength of 70 mM and high Ca^{2+} concentration (Table 1). In accordance with the binding

Table 1: Binding Constants for the Affinity of Different Tropomyosins for F-Actin[a]

	K_d (μM)					
	Omp T-digested tropomyosin (residues 7−284)		carboxypeptidase-treated tropomyosin (residues 1−273)		tropomyosin (residues 10−284)	
ionic strength	EGTA	Ca^{2+}	EGTA	Ca^{2+}	EGTA	Ca^{2+}
70 mM	0.26 ± 0.03	1.65 ± 0.28	0.16[b]	0.34[b]		
163 mM					1.4[c]	N/A[c]
KCl	1.3 ± 0.14	N/A				
KAc	0.32 ± 0.00	2.6 ± 0.6				
~20 mM	0.19 ± 0.01	0.83 ± 0.11				

[a] Summary of K_d values for the binding of different shortened tropomyosins to F-actin. The values for Omp T-digested tropomyosin are from this work. N/A denotes that saturation binding was not attained within the protein concentration range that was used. [b] From ref *10*. [c] From ref *18*.

isotherm (Figure 4, inset) proteins were mixed in a 7:4:2 molar ratio (F-actin:Omp T-digested tropomyosin:troponin).

The thin filament concentration dependence of the steady-state hydrolysis rate is presented in the main part of Figure 3. In this experiment, the concentration of myosin-S1 was kept constant at 0.25 μM while that of actin (either unregulated or regulated) was varied, such that there is a >250-fold molar excess of actin monomer over myosin-S1 by the end of the titration. Under these conditions, thin filaments (with Ca^{2+}) provide less activation than unregulated actin (in Figure 4, compare dashed and solid lines). At the same time, it is apparent that the truncated system generates ~2-fold greater activation than the intact system (Figure 3). A fit of each set of data points to a hyperbola yields maximum rates of 6.8 s^{-1} (intact tropomyosin) and 16 s^{-1} (tropomyosin residues 7−284) at saturating thin filament concentrations. Conversely, there is little, if any, difference in myosin binding affinity ($K_d \sim 30$ μM). Similar values for V_{max} (7.1 and 14.3 s^{-1}) are obtained by double-reciprocal analysis (data not shown). It should be noted that comparison was carried out on the same day. Further, some of the points in the titration were repeated using a 5:7 tropomyosin (Omp T-cleaved or native):actin molar ratio, and a similar trend was observed (data not shown). Also, the composition of thin filaments sedimented by centrifugation was analyzed by SDS−PAGE and was found to be consistent with the binding analysis obtained with radiolabeled protein (Figure 3, inset). Taken together, the results are ascribed to a real difference and not to incomplete reconstitution. This important concern is also addressed by the experiments described below.

The effect which amino-terminal truncation of tropomyosin has on thin filament cooperativity was investigated by myosin-S1 titration in the presence of either ATP or ADP. In the former case (*26, 39−41*), the concentration of myosin-S1 was increased from 1 to 8 μM while the concentrations of actin, tropomyosin (Omp T-digested or native), and troponin were kept constant at 4, 2.3, and 1.15 μM, respectively. Under these conditions, the steady-state MgATPase hydrolysis rate observed in the presence of unregulated actin is linearly dependent upon the amount of S1 (Figure 4A). In the presence of regulatory proteins and added EGTA, inhibition was observed at all concentrations of S1 tested (0−8 μM). There is virtually no difference in the two inhibited rates, as noted in Figure 4. Upon addition of Ca^{2+}, the rates for both systems increase in a nonlinear manner as a function of the myosin concentration (Figure 4A). Over most of the titration, truncated thin filaments are more activating than the integral control. This is reflected in the position in the titration at which the Ca^{2+}-activated rate intersects the unregulated rate. Crossover occurs at a lower myosin-S1 concentration for the truncated system [~2.5 μM vs ~4.5 μM (Figure 5A)]. However, as the titration proceeds to the highest myosin:actin ratios, the disparity between the two activation rates diminishes (Figure 4A). The convergence is clearly demonstrated when these data are plotted as specific activity versus myosin-S1 concentration. The relationship between rate and concentration is steeper in the case of the integral system (Figure 4B). It is evident, therefore, that thin filaments respond less cooperatively to an increasing myosin-S1 concentration when the amino-terminal hexapeptide of the constituent tropomyosin is missing.

The interaction of myosin-S1-ADP with thin filaments (Figure 5) was measured by assaying the ammonium ATPase activity in the supernatant resulting from a high-speed spin. To guard against any contaminating myokinase activity, thin filaments were incubated in the presence of the inhibitor, Ap5A, prior to the sedimentation step. In the case of unregulated F-actin, the isotherm conforms to a hyperbolic relationship in which half-maximal saturation occurs at a ligand concentration of ~0.25 μM (see the dotted line in Figure 5). Isotherms corresponding to the interaction of the ligand with thin filaments deviate from that of unregulated actin (Figure 5), indicating that F-actin is indeed saturated by tropomyosin (either native or Omp T-digested) and troponin. At high pCa (with EGTA), the isotherms, both of which are sigmoidal, are clearly nonsuperimposable. As expected, the relationship between binding and ligand concentration exhibits a steeper increase in the case of integral thin filaments. However, it is the truncated system that shows the higher affinity for the ligand. The magnitude of the difference at the midpoint is 0.1−0.2 of a pS1-ADP unit [apparent K_d values of 0.37 μM (native) and 0.27 μM (truncated)]. Addition of Ca^{2+} increases the affinity of both types of thin filaments for myosin-S1-ADP, and the isotherms superimpose (K_d values of ~0.16 μM). In this instance, the strengthened binding may have precluded detection of a difference in affinity, which is apparent at high pCa.

DISCUSSION

The interaction between the striated muscle thin filament proteins troponin-T and tropomyosin has been evaluated using a processed form of tropomyosin which lacks the first six amino acids and which can be reconstituted into thin filaments at low and high concentrations of Ca^{2+} (Figures 1, 3 (inset), 4, and 5).

Previous research has established that the tail section of troponin-T spans a considerable proportion of the carboxyl-terminal half of tropomyosin (*7−12*), but there are also reports to indicate that the tail extends beyond the overlap

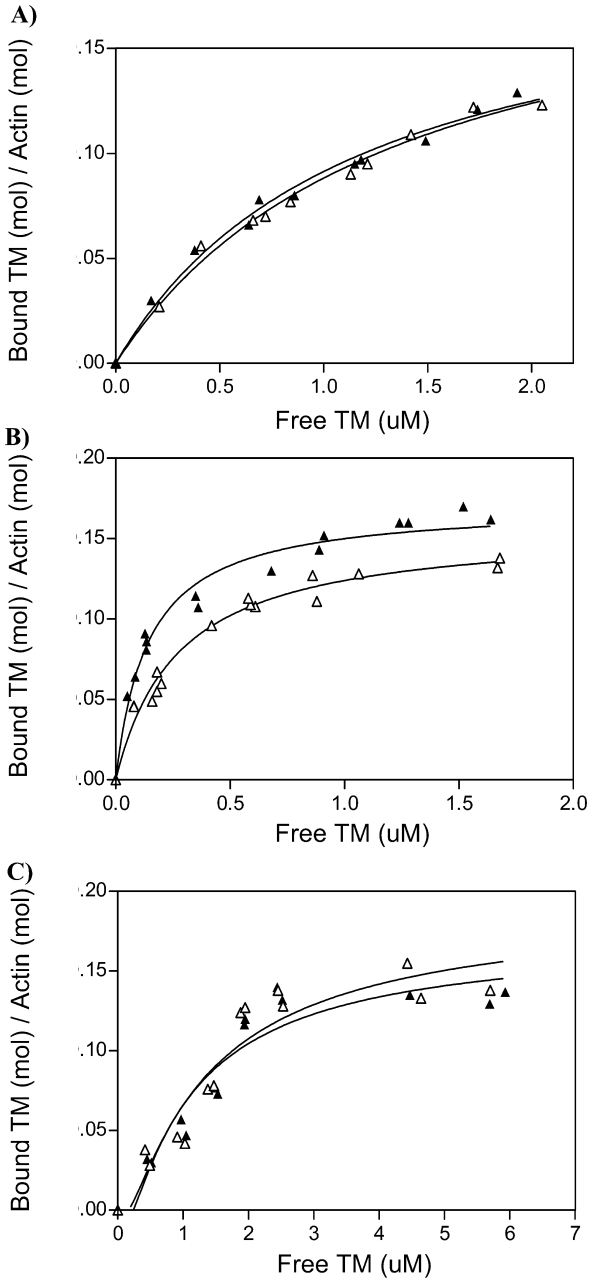

FIGURE 2: Effects of troponin-I and troponin-T fragment, N-Tn-T, on the binding of Omp T-digested, and unacetylated, tropomyosin to F-actin. The buffer consisted of 10 mM MOPS, 50 mM KCl, 5.5 mM $MgCl_2$, and 1 mM DTT (pH 7). Actin and troponin subcomponents were kept constant at concentrations of 7 and 2 μM, respectively, while the concentration of tropomyosin (either Omp T-digested or unacetylated) was varied. The source of the tropomyosin is rabbit skeletal muscle unless otherwise stated. Centrifugation was performed at 4 °C in a TLA 100.2 (Beckman) ultracentrifuge at 150000*g* for 45 min. Curves were fit using the equation $Y = B_{max}[X/(K_d + X)]$ (GraphPad Prism). (A) Actin with Omp T-digested tropomyosin and Tn-I in the absence (▲) and presence (△) of N-Tn-T (residues 1−158). (B) Actin with unacetylated salmon fast skeletal muscle tropomyosin (residues 1−284) and Tn-I in the absence (▲) and presence (△) of N-Tn-T (residues 1−158). (C) Actin with Omp T-digested salmon fast skeletal muscle tropomyosin (residues 7−284) and Tn-I in the absence (▲) and presence (△) of N-Tn-T (residues 1−158).

site to the next tropomyosin molecule in the filament (*24, 25*). Support for such a scenario is provided by the current study. First, N-Tn-T adds to the effect of troponin-I in promoting association of unacetylated tropomyosin (Figure 2B) and

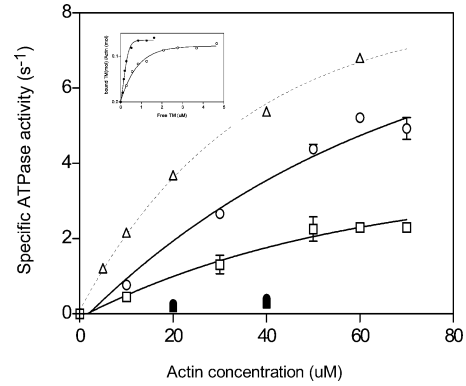

FIGURE 3: Thin filament concentration dependence of the steady-state actomyosin MgATPase activity. Thin filaments were reconstituted separately with either Omp T-digested or full-length tropomyosin. The buffer consisted of 10 mM MOPS, 4.5 mM $MgCl_2$, 1 mM DTT (pH 7), and 0.5 mM EGTA (filled symbols) or Ca^{2+} (empty symbols) with an ionic strength of 20 mM at 25 °C. The concentration of myosin-S1A1 was 0.25 μM. Protein mixtures, containing a supplement of 2 mM $MgCl_2$, were incubated at 4 °C for 30 min. The tubes were then transferred to a 25 °C water bath, and the reaction was initiated by addition of 2 mM ATP. The titration was conducted with a 7:4:2 actin:tropomyosin (either shortened or native):troponin molar ratio. Activities were corrected for the rate of S1 alone. Data shown are representative of at least two experiments. The dotted line shows the rate with unregulated actin. Values of V_{max} by double-reciprocal analysis are 7.1 and 14.3 s^{-1}. Curves were fit using the equation $Y = B_{max}[X/(K_d + X)]$ (GraphPad Prism). The inset shows incorporation of Omp T-digested tropomyosin into thin filaments at an ionic strength of 26 mM. The buffer consisted of 10 mM MOPS, 5 mM KCl, 5.5 mM $MgCl_2$, and 1 mM DTT (pH 7): (filled symbols) with 0.5 mM EGTA and (empty symbols) with 0.5 mM Ca^{2+} at 20 °C. Actin and troponin concentrations in each experiment were kept constant at 7 and 2 μM, respectively, while tropomyosin (residues 7−284) was varied in concentration. K_d values were 0.2 μM (EGTA) and 0.9 μM (Ca^{2+}).

carboxypeptidase-digested tropomyosin (*10*) to F-actin but not Omp T-digested tropomyosin (Figure 2A,C). Second, compared to the native protein, both Omp T-digested tropomyosin and unacetylated tropomyosin, which is structurally disordered at the amino-terminal end (*45*), have weakened affinity for N-Tn-T (*23*). Third, thin filaments composed of Omp T-digested tropomyosin provide greater Ca^{2+} activation of myosin than the fully integral system (Figures 3 and 4). Since N-Tn-T is a tropomyosin-dependent inhibitor (*16, 42*), which stabilizes a turned-off thin filament state (*43, 44*), this observation is consistent with the cleavage having disrupted the tropomyosin−N-Tn-T interaction. A loss of inhibition, and therefore a shift in equilibrium toward a turned-on thin filament state, can also account for the difference in S1-ADP binding to thin filaments (Figure 5). If the various pieces of evidence are combined, N-Tn-T's regulatory role can be proposed to involve attachment to a region of tropomyosin that includes, or is upstream of, residues 1−6. This proposal brings into focus sequence heterogeneity within troponin-T, especially that which is proximal to the amino terminus (*45*−*48*). Conceivably, such diversity could produce isoforms with varying abilities to bridge the overlap and therefore differing regulatory properties. A direct interaction between the amino-terminal portion of tropomyosin and the tail of troponin-T would not preclude the same part of tropomyosin from assisting indirectly in the attachment of the tail to the adjoining tropomyosin (and vice versa). Nor does this work distinguish between different

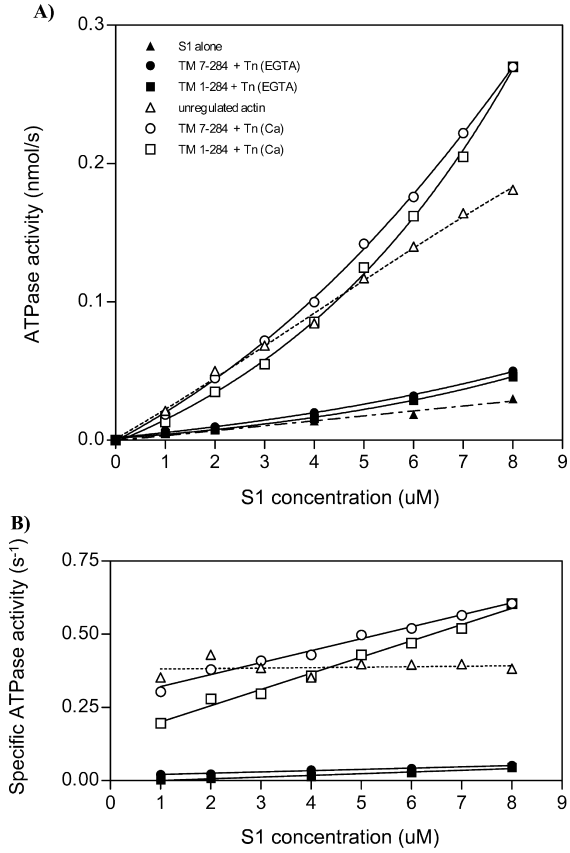

FIGURE 4: Myosin-S1 concentration dependence of regulated actomyosin MgATPase activity. Thin filaments were reconstituted separately with either Omp T-digested or native tropomyosin. Mixtures contained 4.0 μM F-actin, 2.3 μM tropomyosin (either shortened or native), and 1.2 μM troponin. The concentration of myosin-S1A1 was varied. The buffer consisted of 10 mM MOPS, 4.5 mM $MgCl_2$, 1 mM DTT (pH 7), and 0.5 mM EGTA or Ca^{2+}, at 25 °C. (A) MgATPase activity as a function of myosin-S1 concentration. (B) Data from panel A corrected for the rate of myosin-S1 alone and expressed as specific activity. Curves were fit using the equation $Y = B_{max}/[1 + 10^{(\log K_d - \log X)n}]$ (GraphPad Prism). The experiment was repeated more than once with similar results.

models of regulation (*49*−*51*). An alternative explanation for the findings in Figures 3 and 4 is that the amino-terminal region of tropomyosin could itself be inhibitory. However, the above region bares no homology with any known actin-binding site (*17*).

In many respects, the current study is a companion to those involving carboxypeptidase-digested tropomyosin. Both truncated proteins require troponin (Figures 1 and 3, inset, and refs *9* and *10*) to bind to F-actin in the micromolar range, and the resulting thin filaments exhibit only residual cooperativity (Figures 4 and 5 and refs *10* and *27*). The latter would appear to arise, in large part, from the discontinuity in tropomyosin since the product of carboxypeptidase digestion is intact at the amino-terminal end. Nonetheless, on the basis of our results and previous findings (*24, 25*), it is still reasonable to assume that the attachment of troponin-T to contiguous tropomyosins is part of the physical continuity of the thin filament.

Conversely, these nonintegral thin filaments are easily distinguished from each other in assays that involve myosin. Amino-terminal processing (six amino acids subtracted) leads to greater S1 activation (Figure 3) and S1-ADP affinity

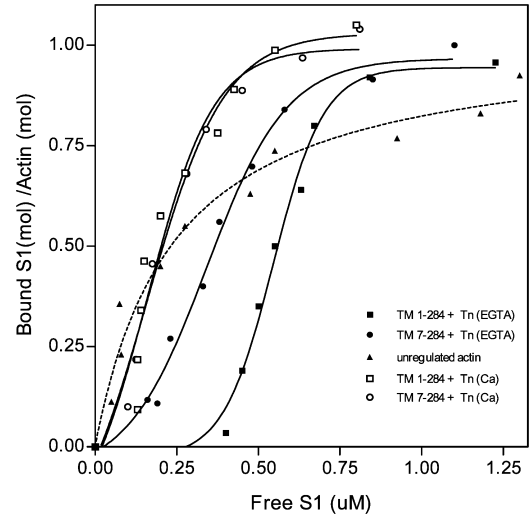

FIGURE 5: Binding of myosin-S1-ADP to thin filaments containing either Omp T-digested or native tropomyosin. Mixtures contained 4.0 μM F-actin, 2.3 μM tropomyosin (either digested or native), 2.3 μM troponin (0.5 mM EGTA or Ca^{2+}), 20 μM myokinase inhibitor, and 0−5 μM myosin-S1A1. The buffer consisted of 10 mM imidazole, 50 mM KCl, 5.5 mM $MgCl_2$, 3 mM MgADP, 1 mM DTT (pH 7), and 0.5 mM EGTA or Ca^{2+}. The supernatant resulting from centrifugation for 30 min at 150000*g* in an Airfuge (20 °C) was subjected to an NH_4/EDTA ATPase activity assay to determine free S1. The supernatant (30 μL) was first mixed with 255 μL of buffer [0.44 M NH_4Cl, 30 mM EDTA, and 28 mM Tris (pH 8.0)]. Reaction was initiated by addition of 15 μL of 100 mM ATP. A standard curve was constructed by assaying known quantities of myosin-S1A1. There was no detectable sedimentation of myosin-S1A1 in the absence of other proteins. The dotted line shows data for unregulated actin. The fit is to the hyperbolic relationship $Y = B_{max}[X/(K_d + X)]$. Other curves are fit to the equation $Y = B_{max}/[1 + 10^{(\log K_d - \log X)n}]$.

(Figure 5) relative to the control, whereas carboxyl-terminal processing (11 amino acids subtracted) has the reverse effects (*26, 27*). The different outcomes associated with shortening the molecule at one of its two ends suggest that the end regions of tropomyosin, in conjuction with the other regulatory components, serve opposing regulatory roles.

In summary, this study sheds light on the structural basis of the modulatory activity of the troponin-T tail. The case for the physical linkage of two successive molecules of tropomyosin by troponin-T continues to grow.

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