Distinct Regions of Troponin I Regulate Ca\(^{2+}\)-dependent Activation and Ca\(^{2+}\) Sensitivity of the Acto-S1-TM ATPase Activity of the Thin Filament*

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The regions of troponin I (TnI) responsible for Ca\(^{2+}\)-dependent activation and Ca\(^{2+}\) sensitivity of the actin-myosin subfragment 1-tropomyosin ATPase (acto-S1-TM) activity have been determined. A colorimetric ATPase assay at pH 7.5 has been applied to reconstituted skeletal muscle thin filaments at actin:S1:TM ratios of 6:1:2. Several TnI fragments (TnI-(104–115), TnI-(1–116), and TnI-(96–148)) and TnI mutants with single amino acid substitutions within the inhibitory region (residues 104–115) were assayed to determine their roles on the regulatory function of TnI. TnI-(104–115) is sufficient for achieving maximum inhibition of the acto-S1-TM ATPase activity and its importance was clearly shown by the reduced potency of TnI mutants with single amino acid substitutions within this region. However, the function of the inhibitory region is modulated by other regions of TnI as observed by the poor inhibitory activity of TnI-(1–116) and the increased potency of the inhibitory region by TnI-(96–148). The regulatory complex composed of TnI-(96–148) plus troponin T-troponin C complex (TnTC) displays the same Ca\(^{2+}\) sensitivity (pCa\(^{50}\)) as intact troponin (Tn) or Tn plus TnTC while those regulatory complexes composed of TnTC plus either TnI-(104–115) or TnI-(1–116) had an increase in their pCa\(^{50}\) values. This indicates that the Ca\(^{2+}\) sensitivity or responsiveness of the thin filament is controlled by TnI residues 96–148. The ability of Tn to activate the acto-S1-TM ATPase activity in the presence of calcium to the level of the acto-S1 rate was mimicked by the regulatory complex composed of TnI-(1–116) plus TnTC and was not seen with complexes composed with either TnI-(104–115) or TnI-(96–148). This indicates that the N terminus of TnI in conjunction with TnTC controls the degree of activation of the ATPase activity. Although the TnI inhibitory region (104–115) is the Ca\(^{2+}\)-sensitive switch which changes binding sites from actin-TM to TnC in the presence of calcium, its function is modulated by both the C-terminal and N-terminal regions of TnI. Thus, distinct regions of TnI control different aspects of TnI's biological function.

Muscle contraction occurs through the interaction between myosin and the thin filament which is composed of actin, tropomyosin (TM), and troponin (Tn). The Tn regulatory complex consists of three proteins: troponin I (TnI), the inhibitory protein; troponin T (TnT), which binds to TM; and troponin C (TnC), which binds Ca\(^{2+}\). In the absence of Ca\(^{2+}\), TnI interacts with actin-TM and inhibits the acto-S1-TM ATPase activity while in the presence of Ca\(^{2+}\), TnI interacts with TnC and the inhibition is released. Full biological function which includes inhibition, Ca\(^{2+}\)-dependent release (neutralization), activation of the ATPase activity, and Ca\(^{2+}\) sensitivity requires all three Tn components. The activation of the acto-S1-TM ATPase activity to the acto-S1 ATPase rate (unregulated actin) can occur in the presence of Tn and Ca\(^{2+}\) or at high ratios of S1 to actin by TM alone (1). Full regulation by Tn may be due to changes in the interactions between TnI and actin, TM, TnT, or TnC. Therefore, it is critical to identify and determine the contributions of different regions of TnI toward the various aspects of Tn regulation.

Studies of skeletal TnI, using synthetic peptides, proteolytic and/or recombinant fragments have identified several regions of TnI that interact with both actin-TM and TnC (2–4). Syska et al. (5) demonstrated that the cyanogen bromide fragment of skeletal TnI, residues 96–116, possessed all the inhibitory properties of TnI. In fact, a synthetic peptide, TnI-(104–115), comprised the minimum sequence required for the maximum level of inhibition of the actomyosin or actomyosin fragment S1 ATPase activity (4, 6, 7). TnI-(104–115) binds TnC resulting in the Ca\(^{2+}\)-dependent release of inhibition (8, 9). As well, TnI-(104–115) has been shown to replace intact TnI in regulating the Ca\(^{2+}\)-dependent contraction and relaxation of skinned muscle fibers.

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muscle fibers (10). There are other regions within TnI that are also known to modulate TnI function. For example, the N-terminal synthetic peptide TnI(1–40) binds to TnC and prevents the Ca²⁺-dependent release of TnI or TnI(104–115) inhibition (9). A TnI mutant in which residues 1–57 are deleted, TnI(del57), has a modified interaction with TnC and does not interact with TnT (11, 12). The N terminus of skeletal TnI is critical for the incorporation of TnI into the ternary complex with TnT and TnC. In addition, the C terminus of TnI in conjunction with the inhibitory region is critical for the Ca²⁺-dependent reconstitution of Tn activity in actomyosin ATPase assays (13). TnI(96–116) displays an 8-fold increase in affinity for TnC compared with TnI(104–115) (14) and by extending the sequence toward the C terminus TnI(96–148) displays an even larger increase in the binding affinity for TnC (15).

The present study was undertaken to determine the effect of the N- and C-terminal regions of skeletal TnI (situated adjacent to the inhibitory region 104–115) on the Ca²⁺-dependent control of the acto-S1-TM ATPase activity and their ability to modulate this inhibitory region. Recombinant TnI fragments corresponding to residues 1–116 (TnI(1–116)) and residues 96–148 (TnI(96–148)), a synthetic peptide corresponding to residues 104–115 (TnI(104–115)), and TnI mutants with single amino acid substitutions within the inhibitory region (TnI(K105G) and TnI(L111G)) display different binding affinities for actin-TM and/or the complex of tropinin T and tropinin C (TnTC). Therefore, we designed a general protocol for the reconstitution and analysis of regulatory complexes composed of TnI mutants or TnI fragments. This protocol allows for rapid and easy evaluation of TnI fragments or mutants showing different affinities for either actin-TM and/or TnT and TnC. The manipulation of the molar ratio of the TnI fragments or mutants with respect to the other thin filament proteins allows for the reconstitution of a functional regulatory complex in the acto-S1-TM ATPase assay. Under the conditions of maximum Ca²⁺-response, the composition of the various regulatory complexes reconstituted on the thin filament are similar, even though the concentrations in solution may differ. We have found that the N- and C-terminal regions of TnI work in conjunction with TnT-C to influence different regulatory aspects of the reconstituted thin filament. That is, the C-terminal region of TnI plays a role in controlling the Ca²⁺ sensitivity or responsiveness of the thin filament and the N-terminal region of TnI is critical for activation of the ATPase activity.

MATERIALS AND METHODS

Preparation of Proteins, Peptides, and Assay Buffers—Rabbit skeletal muscle Tn was purified according to the protocol of Ebashi et al. (16) with the following modifications: the wash buffer contained 20 mM KCl and 2 mM potassium carbonate; the extraction buffer contained 0.6 M lithium chloride and 50 mM sodium acetate, pH 4.5; contaminating TM was removed by isoelectric precipitation, pH 4.5; and the ammonium sulfate cuts were 0–40, 40–50, and 50–60%. Rabbit skeletal troponin T (TnT) and rabbit skeletal tropocin C (TnC) subunits were isolated according to the protocol of Pan et al. (17) with minor modifications; the column buffer contained 8 mM urea, 50 mM Tris, 5 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride; and the gradient was 0–0.5 M sodium chloride. Rabbit skeletal troponin I (rTnI) was further purified according to Wilkinson (18) with the following modifications: the column buffer contained 8 mM urea, 100 mM sodium acetate, and 1 mM dithiothreitol, pH 4.5; and the gradient used was 0–25 M sodium chloride. Recombinant rabbit skeletal muscle TnI (rrTnI) was expressed as described previously (19) and the TnI(K105G) and TnI(L111G) mutants were prepared according to Strauss et al. (20). Rabbit skeletal muscle recombinant TnI (rrTnI) and TnI(1–116) were prepared and purified according to Farah et al. (13). Chicken skeletal recombinant TnI(96–148) was prepared according to Pearlstone et al. (15). The synthetic TnI peptide, TnI(104–115), was synthesized and purified by HPLC as described by Van Eyk and Hodges (4). The purity and authenticity of the peptides or fragments were verified by amino acid analysis and mass spectrometry.

Skeletal actin was prepared according to Spudich and Watts (21) and the final polymerization was carried out in either 6.5 mM KCl/ATPase buffer or 100 mM KCl binding buffer as described below. Cardiac troponin was prepared according to Smillie (22). Skeletal myosin subfragment I (S1A1/A2) was prepared according to Taylor (23). The potassium-EDTA ATPase activity of the S1 was 7.2 nmol of phosphate released s⁻¹ mol⁻¹ S1. Myosin S1(A1/A2) and TM were dialyzed against 6.5 mM KCl/ATPase buffer. The potassium chloride (KCl) concentrations of the assay buffers were varied during the protein preparation and for different ATPase experiments. Therefore, when listing the ATPase buffers used for the experiments, the KCl concentration is listed in parentheses. For example, the ATPase (6.5 mM KCl) buffer refers to the assay buffer consisting of 20 mM Tris, 6.5 mM KCl, 3.5 mM MgCl₂, 0.5 mM EGTA, 1 mM dithiothreitol, and 0.01% sodium azide (pH 7.8) while the binding buffer (100 mM KCl) contained 20 mM Tris, 100 mM KCl, 5.0 mM MgCl₂, 0.5 mM EGTA, 1 mM dithiothreitol, and 0.01% sodium azide (pH 7.8). The KCl concentration for the ATPase assay was 6.5, 13, or 19 mM for 320 µl or 19 mM KCl for a 200-µl assay volume. The salt concentration of the assay buffer or the assay volume did not affect the relative acto-S1-TM ATPase activity, the pCa₅₀ or n₅₀ values of the ATPase/pCa curves (Table I) but did change the absolute ATPase activity. The buffers used to dissolve and dialyze Tn, TnC, or the TnTC complex contained 50 µM CaCl₂ and no EGTA and are identified as ATPase (6.5 mM KCl) buffer minus EGTA buffer.

Preparation of Tn, TnT, and Individual Tn Subunits—TnT and TnC were dissolved separately in ATPase (1 M KCl) minus EGTA buffer containing 6 mM urea at approximately 5–10 mg/ml. The TnT and TnC were then dialyzed against ATPase (1 M KCl) minus EGTA buffer containing 2 mM urea. TnT and TnC were combined in a 1:1 molar ratio based on peak area obtained from the elution profile at 210 nm from reversed-phase high performance liquid chromatography (for more details, see the centrifugation studies section). The TnTC complex was subsequently dialyzed against the following series of buffers consisting of ATPase minus EGTA buffer containing 1, 0.5, 0.25, and 0.1 mM KCl. TnT was further dialyzed to 6.5 mM KCl when TnTC was used to reconstitute a regulatory complex with rrTnI, TnI(K105G), or TnI(L111G). The mole ratio of TnT to TnC in the final solution ranged between 1.15 and 1.18. This is the mole ratio required to keep TnT in solution when the concentration of KCl is 1 mM and below. All TnTC concentrations listed in the article are based on the TnT concentration.

CrTnI, TnI(1–116), rrTnI, TnI(L111G), and TnI(K105G) were prepared using the same dialysis procedure as described for the TnTC complex except the ATPase buffer contains 0.5 mM EGTA and no CaCl₂. The final dialysis buffer for rrTnI, TnI(K105G), TnI(L111G), and CrTnI was ATPase (100 mM KCl) buffer and for TnI(1–116) the final buffer was ATPase (6.5 mM KCl) buffer. Rabbit skeletal TnI was either prepared in the same manner as the two recombinant TnI proteins (final buffer ATPase (100 mM KCl) with final concentration of approximately 1 mg/ml) or dissolved directly in H₂O (pH 3–5.5) with a final concentration of approximately 4 mg/ml. Due to the higher solubility of TnI at low pH, the volume of TnI added to the assay was small and did not alter the pH of the assay buffer. TnI(104–115), TnI(96–148), and rabbit skeletal Tn were dissolved directly in ATPase (6.5 mM KCl) buffer or binding (100 mM KCl) buffer. Prior to use, the proteins and peptides were either centrifuged at 15,000 rpm at 4 °C for 5 or 30 min if they were to be used in the centrifugation experiments. The concentrations of the proteins, peptides, and fragments were determined by amino acid analysis except for S1 which was determined by absorbance.

ATPase Assays—A colorimetric method was used to quantify the amount of Pₐ liberated by myosin S1. The colorimetric method is based on Heinonen and Lahti (24) except Tris was used in the ATPase buffer and the pH increased from 7.0 to 7.8. The assay volume was either 200 or 320 µl. The amount of protein added to each assay volume was: 1.5 nmol of F-actin, 0.25 nmol of S1, and 0.429 nmol of TM (dimeric). The reaction was preincubated for 10 min at 25 °C and then initiated with the addition of ATP (2 mM). The reaction was allowed to proceed for 10–35 min depending on the assay. Control experiments demonstrated that the ATPase rate under these conditions were linear. The reaction was stopped and the quantity of liberated inorganic phosphate (Pi) was determined by the addition of 2 M of a 24% solution of sulfuric acid, 10 mM ammonium molybdate solution followed by the addition of 240 µl of a 1 M citric acid solution. The absorbance of the individual tubes were determined at 355 nm. The endogenous S1 ATPase rate was subtracted from the data (i.e. 0.065 nmol phosphate released s⁻¹ mol⁻¹ S1, 6.5 mM KCl, 320-µl assay volume). Each ATPase data point was determined in triplicate. In addition, each pCa
versus ATPase curve was repeated twice. The standard deviation between these curves were plotted.

In the inhibition assays, increasing quantities of TnI or TnI fragments were added until maximum inhibition of the acto-S1-TM ATPase activity was reached. For the Ca\(^{2+}\)-dependent release assays the concentration of inhibitor required to reach maximum inhibition was used and increasing quantities of a performed TnC complex or TnC was added until maximum Ca\(^{2+}\) activation of the acto-S1-TM ATPase activity was achieved. In the presence of 0.429 nmol of Tn (mole ratio of 1:1 with TM) the acto-S1-TM ATPase activity was determined in both the absence (pCa 8.0) and presence of Ca\(^{2+}\) (pCa 3.5). The Ca\(^{2+}\) versus ATPase curves were obtained using the concentration of TnTC and TnI, TnI fragments, or TnI mutants that induce the maximum Ca\(^{2+}\)-dependent ATPase activity. Increasing quantities of 2.64 or 5.40 mM standardized calcium chloride was added to achieve pCa values between 3.5 and 9.0. Precipitation occurred with TnI-(104–115) and TnI-(1–116) unless there was a low concentration of Ca\(^{2+}\) (pCa ~6.9) present in the assay prior to the addition of the TnC complex. At this low Ca\(^{2+}\) concentration, the ATPase activity was similar for the regulatory complex and the TnC, TnI, or TnI-(1–116) in the presence and absence of the TnC (Fig. 4, Table I). The increase in the volume did lower the overall turnover time of the complex due to the decrease in the concentration of S1 and the individual thin filament components (compare the acto-S1-TM ATPase activities of 0.455 ± 0.015 to 0.656 ± 0.004 nmol of phosphate released s\(^{-1}\) nmol\(^{-1}\) S1 in 320 and 200 m at 6.5 mM KCl, respectively).

The precautionary measures taken to control the calcium concentration have been described (25). Free calcium concentrations were calculated taking into account ionic strength and pH as described by Chandras et al. (14). The log of the individual binding constants used in the present work were as follows: EGTA + H = EGTA-H, 9.526; EGTA-H + H = EGTAH-H, 18.454; EGTA-H2 + H = EGTAH2-H, 21.254; EGTA3-H + H = EGTAH4-H, 23.374; EGTA + Ca = EGTA-Ca, 11.310; EGTAH + Ca = EGTAH-Ca, 14.856; TnC + 2Ca = TnCCa\(_2\), 3.400; TnCCa\(_2\) + 2Ca = TnCCa\(_4\), 24.806; EGTAH + Mg = EGTAH-Mg, 7.000; EGTAH + Mg = EGTAH-Mg, 12.896; ATP + H = ATP-H, 7.067; ATP + H = ATP-H2, 4.140; ATP-H2 + H = ATP-H3, 1.000; ATP-H3 + H = ATP-H4, 1.000; ATP + Ca = ATP-Ca, 4.158; ATPH + Ca = ATPH-Ca, 8.851; ATP + Mg = ATP-Mg, 4.422; ATP-H + Mg = ATP-HMg, 9.831. The Ca\(^{2+}\) ATPase data were curve fitted to a sigmoidal curve using the calculus graph program, Aplo, and Macrojax.0

Centrifugation Studies—To quantitate the amount of each regulatory protein bound to the actin thin filament, binding studies were carried out in binding (100 mM KCl) buffer in the presence of 3 mM CaCl\(_2\). The concentration of MgCl\(_2\) (26–29) and KCl (26) were increased compared with the ATPase buffer (5 and 100 mM, respectively) to ensure maximum binding of TM in the absence of Tn. In addition, the higher salt concentrations (100 mM KCl) prevented nonspecific binding of proteins to the centrifuge tubes and ensured quantitative recovery of the proteins. Since lower salt concentrations were used in the ATPase assays than in the binding experiments, it is important to note that the composition of the various regulatory complexes bound to actin-TM in the ATPase assays may be different than that observed in the binding experiments. The total assay volume used was 175 μl. The concentration of actin was 1.3 μM and the mole ratio of actin to TM was 7:2. Actin, TM, Tn, TnI-(1–116), and TnTC were dialyzed against the binding buffer. Tn, TnI, or TnI-(1–116) in the presence and absence of the TnTC were added to the actin and TM mixture. The samples were spun for 30 min at approximately 24,000 g on a Beckman airfuge using a 18 A-100 rotor. This resulted in 90–99% of the actin being pelleted. The pellets were dissolved in 50 μl of 0.05% aqueous trifluoroacetic acid. 40 μl of this solution was injected on a Zorbax CB 350 reversed-phase column (4.6 mm inner diameter × 250 mm) on a Hewlett Packard series 1090 LC coupled to a Hewlett Packard Vectra 48166 XM processor. The various proteins and fragments were eluted using a 2% B/min linear gradient where eluent A is 0.05% aqueous trifluoroacetic acid and eluent B is 0.05% trifluoroacetic acid in acetonitrile at a flow rate of 1 ml/min (40). The peak areas were determined at 210 nm and/or 280 nm and converted to nanomoles using a standard curve obtained for each protein or fragment. The standard deviation of the standard curves ranged from 5 to 8%. The amount of protein (TM, Tn, TnC, and TnI or TnI fragment pelleted in the absence of actin (2–7.5%) was subtracted from the amount pelleted in the presence of actin. The experiments were done in triplicate and the standard deviations were calculated.

RESULTS

Inhibitory Action of Skeletal TnI and TnI Fragments on the Acto-S1-TM ATPase—All of the skeletal TnI fragments except TnI-(1–116) were able to inhibit the acto-S1-TM ATPase activity to approximately the same degree as native skeletal muscle TnI (10%, Fig. 1) or intact Tn in the absence of calcium (18%, data not shown). However, the quantity required to achieve this level of inhibition markedly differed between the various skeletal TnI fragments (Fig. 1). For example, compared with rabbit skeletal TnI, TnI-(104–115) is a less effective inhibitor requiring approximately three times the concentration to reach maximum inhibition (compare IC\(_{50}\) values of 0.84 to 2.25, respectively; Table I). The importance of the inhibitory region (residues 104–115) is also illustrated by comparing rtTnI to either TnI (K105G) or TnI(L111G). These mutants require a 2.2–3-fold higher concentration to induce the same maximum level of inhibition of the acto-S1-TM ATPase activity (compare the IC\(_{50}\) of 0.62 with 1.88 and 1.37, respectively; Table I). This indicates that the single amino acid substitution of lysine at position 105 or leucine at position 111 with glycine in the intact protein reduces its apparent affinity for actin-TM. The frag-
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TABLE I

Comparison of the biological function of the various regulatory complexes

| Protein composition of regulatory complexa | \( n_H \) \( b \) | pCa\( _{50} \) \( c \) | IC\( _{50} \) \( d \) | Maximum Ca\( ^{2+} \) ATPase\( e \) |
|------------------------------------------|----------------|----------------|----------------|-----------------|
| A, salt concentration and assay volumesf | \( \mu M \) | % | | |
| Rabbit skeletal Tn (2:2:7, low salt, 200 \( \mu l \)) | 3.5 \( \pm \) 0.3 | 6.12 \( \pm \) 0.01 | | |
| Rabbit skeletal Tn (2:2:7, high salt, 200 \( \mu l \)) | 2.9 \( \pm \) 0.8 | 6.11 \( \pm \) 0.04 | | |
| Rabbit skeletal TnT \( \cdot \) C \( \cdot \) I (2:2:7, low salt, 200 \( \mu l \)) | 4.0 \( \pm \) 0.4 | 6.15 \( \pm \) 0.01 | | |
| TnC \( \cdot \) T plus rabbit skeletal Tnl (6:2:7, high salt, 200 \( \mu l \)) | 4.7 \( \pm \) 0.9 | 6.16 \( \pm \) 0.02 | | |
| TnT \( \cdot 

a The assay consisted of 0.428 mmol of TM (dimeric) and 1.5 mmol of actin (ratio 2:7) and 0.249 mmol S1. The values in brackets show the ratio of skeletal Tnl or Tnl fragment: TnT \( \cdot \) C at a 3:6:7 ratio of TnI:TnT \( \cdot \) C.

b \( n_H \) is the Hill coefficient determined from the ATPase/pCa curves for each regulatory complex determined from Fig. 4.

c pCa\( _{50} \) is the pCa (-log concentration of Ca\( ^{2+} \)) required to induce half of the Ca\( ^{2+} \)-dependent change in ATPase activity. The pCa\( _{50} \) value is determined from the ATPase/pCa curves for each regulatory complex from Fig. 4.

d The IC\( _{50} \) value, the concentration (\( \mu M \)) of inhibitor to induce 50% of the inhibition of the acto-S1-TM ATPase activity, was determined from curve fitting the data in Fig. 1.

e The calculated maximum Ca\( ^{2+} \)-dependent ATPase activity determined from curve fitting the data in Fig. 2 (acto-S1-TM ATPase = 100%). The acto-S1-TM ATPase activity for TnT \( \cdot \) C at a 6:2:7 mole ratio of TnT \( \cdot \) C:TM:actin is approximately 110%.

f The volume of assay or the salt concentration of the assay buffer, 6.5 mM KCl (low salt), or 13 mM KCl (high salt, 320 \( \mu l \)), did not significantly alter pCa\( _{50} \) or IC\( _{50} \) values.

- The Ca\( ^{2+} \)-dependent Release of Inhibition by TnT \( \cdot 

- The Ca\( ^{2+} \)-sensitive regulatory complex composed of TnI-(1–116) plus TnT \( \cdot 

- The N-terminal region of TnI in conjunction with TnTC is required to activate the ATPase activity to rates above acto-S1-TM. Farah et al. (13) showed that a regulatory complex formed by the renaturation of TnI-(1–116), TnT, and TnC at a 1:1:1 molar ratio from urea/KCl activates the actomyosin ATPase activity in the presence of Ca\( ^{2+} \) to 103%. This value is similar to the acto-S1-TM ATPase activity induced at a 1:1:1 molar ratio of TnT:C:TnI-(1–116) of 150% (Fig. 2A). The large ratio of TnT:C:TnI-(1–116) (4:1) required for maximal Ca\( ^{2+} \)-dependent activation of the acto-S1-TM ATPase activity (Fig. 2A) and the poor inhibition of the acto-S1-TM ATPase activity by TnI-(1–116) (16% inhibition at a molar ratio of 3:1 TnI-(1–116): TM, Fig. 1A) indicates that additional binding sites in the C-terminal region of TnI for TnT and/or TnC are needed to duplicate the effectiveness of TnI as a whole. The low ratio of TnT:C:TnI-(96–148) required for maximal Ca\( ^{2+} \)-dependent activity (2:1) compared with the high ratios required by TnI-(1–116) and TnI-(104–115) (4:1 and 6:1, respectively) suggests that additional TnI residues within 117–148 are involved in TnT:C binding (Fig. 2A).

Approximately a 1:1 molar ratio of TnT:C to rrTnI, TnI (K105G), or TnI (L111G) is required to reach the maximum Ca\( ^{2+} \)-dependent ATPase activity (Fig. 2B). Interestingly, the regulatory complex composed of TnI (L111G) plus TnT \( \cdot 

Centrifugation Studies—To determine whether differences in efficacy are a result of differences in binding affinity, we determined the composition of the thin filament proteins bound to actin at the various mole ratios of these proteins that were required for maximum inhibition and Ca\( ^{2+} \)-dependent activation of the ATPase activity. Actin centrifugation experiments
were performed under conditions similar to the ATPase assay with the exception that a higher concentration of MgCl₂ and KCl was used to promote binding of TM to actin in the absence of Tn (26–29) as well as to remove nonspecific binding of the proteins to the centrifuge tubes (see “Materials and Methods”). To monitor all of the thin filament proteins simultaneously after centrifugation, the pellet was analyzed by reversed-phase HPLC. Fig. 3 shows sample elution profiles for the pellet obtained from centrifugation studies of actin, TM, and various components of the regulatory complexes. From the HPLC elution profiles, the peak areas of the various proteins or peptides can be determined at 210 or 280 nm and converted to nano-moles using the standard curve for each protein or peptide. The influence of TnT on binding of TnI-(1–116) to actin-TM is also shown in Table II. More TnI-(1–116) is bound to actin in the presence of calcium (pCa 5 3.5) than in the absence of calcium (pCa 5 6.7), TnI (104–115) = 6.7, and TnI (96–148) = 3.7. Panel B shows the effect of increasing quantities of TnC in the presence of calcium (pCa 5 3.5) on the ATPase activity with rrTnI (●), TnI (K105G) (◇), or TnI (L111G) (○). The inhibitors were present at the concentration required for maximum inhibition (mole ratio of TnI or TnI mutant to actin were: rrTnI = 3:7; crTnI = 3:7; TnI-(1–118) = 6.7; TnI-(104–115) = 6.7, and TnI (96–148) = 3:7). Panel B shows the effect of increasing quantities of TnC in the presence of calcium (pCa 5 3.5) on the ATPase activity with rrTnI (●), TnI (K105G) (◇), or TnI (L111G) (○). The inhibitors were present at the concentration required for maximal inhibition (mole ratio of TnI or TnI mutant to actin were: rrTnI = 4:7; TnI (K105G) = 9:7, and TnI (L111G) = 9:7). The actin and TM concentrations were 7.5 and 2.14 μM, respectively. 100% is equal to the acto-S1-TM ATPase rate. The average with the standard deviation of at least three independent determinations is shown. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point. All assays were performed in ATPase (13 mM KCl) buffer in 320 μl as described under “Materials and Methods.”

Although an even higher mole ratio of TnT-C was required for maximum Ca²⁺-dependent release in the ATPase assays for TnI-(1–116) (4:1), Fig. 2A, the amount of the regulatory proteins bound to actin in the centrifugation experiments were similar (experiments 5 and 7, Table II). This means that under conditions of maximum calcium response, the composition of the thin filament is similar for the different regulatory complexes even though their concentrations may differ in solution.

The influence of TnC on binding of TnI-(1–116) to actin-TM is also shown in Table II. More TnI-(1–116) is bound to actin in the presence than in the absence of TnC (experiments 6 and 7 to experiment 3, ratio of 1.34 or 1.27 versus 0.98). A change in the TnC:TM ratio from 1:1 to 1:1.6 ratio from 1:1 to 4:1 (experiments 6 and 7, Table II) did not result in any further increase in TnI-(1–116) bound to actin-TM. The increase in the ATPase activity when the ratio of TnT-C increases from 1:1 to 4:1 (Fig. 2A) is due to an increase in TnC:TM bound to actin-TM. A change in the TnC:TM ratio from 1:1 to 1:1.6 does not affect the amount of TnT-C bound (the same amount of TnT-C is bound when TnI-(1–116) is not present in the assay (compare experiments 7 and 8, Table II)). One cautionary comment should be made concerning these results, although in the absence of calcium, TnI, TnT, and TnC were bound to TM-actin in similar mole ratios as in the presence of calcium, this does not mean that some of the regulatory complexes composed of TnT fragments (which have different affinities for actin and TnC) and TnC may have different ratios bound to actin-TM in the absence of calcium.

**Ca²⁺ Affinity and Ca²⁺-dependent Cooperativity.—** The contribution of TnI and TnI fragments to the calcium affinity (pCa₉₀) and the calcium-dependent cooperativity (α₉₀) of reconstituted thin filaments was determined from the ATPase/pCa relationship. One method for reconstitution is to renature the individual subunits, TnI, TnT, and TnC at a molar ratio of 1:1:1 from urea/KCl slowly through a series of buffers containing no

2 J. E. Van Eyk, L. T. Thomas, and R. S. Hodges, unpublished data.
urea and decreasing concentration of KCl. A regulatory complex reconstituted in this manner (TnT-C:I) has similar pCa50 and nH values to native Tn (Fig. 4A, Table I). However, an alternative method is required when dealing with TnI mutants or fragments that display reduced affinity for TnT, TnC, or actin. We have chosen to reconstitute the regulatory complex by the addition of TnI to the assay solution followed by the addition of a preformed TnT-TnC complex at a mole ratio of these proteins that produces the maximum Ca2+-dependent change in the acto-S1-TM-ATPase activity (Fig. 4A). For example, in Fig. 1B a 1.5:1 mol ratio of TnI to TM is required for maximum inhibition. At this concentration of TnI, a 2:1 mol ratio of TnT-C to TnI (equivalent to a 1.5:3:1 ratio of TnI:TnT-C:TM) is required for maximum ATPase activity (Fig. 2A). At these mole ratios, the regulatory complex behaves like native Tn displaying similar Ca2+-sensitivity (Fig. 4A, Table I). This reconstitution method should also work for TnI fragments which have reduced affinities for TnT-C and actin. To achieve maximum Ca2+ response of the regulatory complex for TnI-(1–116), a molar ratio of 3:12:1 of TnI-(1–116):TnT-C:TM was required (Fig. 4B). Importantly the binding experiments show that the amount of protein bound to actin with the regulatory complexes composed of TnT-C and either TnI or TnI-(1–116) were equivalent, even though the concentration in solution differed. Thus, under these conditions where the various regulatory complexes display maximum Ca2+-change in the acto-S1-TM ATPase activity, the pCa/ATPase relationship should be comparable. In fact, it is the use of the high concentrations of TnI-(1–116) and TnT-C required for full effect and binding which can explain the differences between the ATPase/pCa curve obtained in this study and that reported by Farah et al. (13). The difference lies with the extent of Ca2+-dependent activation by the TnI-(1–116) regulatory complexes which are prepared to have different mole ratios with respect to both TnT-C and actin-TM. Farah et al. (13) reconstituted the regulatory complex from a mixture containing TnI-(1–116), TnT, and TnC at a 1:1:1 mol ratio and the assay contained a 1:1 mol ratio with respect to TM. This regulatory complex displayed no Ca2+-dependent response. In this study, the regulatory complex was reconstituted on the thin filament using high mole ratios of TnI-(1–116) and TnT-C. Under these conditions there was full Ca2+-dependent activation (Fig. 2) and hence a ATPase/pCa curve was observed (Fig. 4).

The reconstitution of the thin filament with native rabbit skeletal TnI, rabbit recombinant or chicken recombinant skeletal TnI plus TnT-C complexes show similar pCa/ATPase relationships with pCa50 values of 6.16, 6.14, and 6.08, respectively, and nH values of 4.7, 3.8, and 3.1, respectively (Table I, part B). TnI-(96–148) plus TnT-C displayed similar Ca2+-sensitivity (pCa50 = 6.01) and cooperativity (nH = 3.1) as that of intact TnI (Fig. 4C). However, the regulatory complexes composed of TnI-(104–115) or TnI-(1–116) plus TnT-C displayed increased Ca2+-affinity (pCa50 = 6.52 and 6.36, respectively) compared with TnI-(96–148) plus TnT-C (pCa50 = 6.01) (Fig. 4B, Table I). The shift in pCa50 of TnI-(1–116) and TnI-(104–115) from TnI-(96–148) is independent of the ability of these fragments to activate the ATPase activity, i.e. both 104–115 and 1–116 shift the pCa50 but only 1–116 activates. Interestingly, the largest difference in pCa50 values occurs between peptides TnI-(104–115) and TnI-(96–148) (both of which do not activate the ATPase activity above the acto-S1-TM rate) suggesting that other residues outside the inhibitory region 104–115 are responsible for the change in Ca2+-sensitivity.

Fig. 4D compares the pCa/ATPase curves obtained for the regulatory complexes composed of TnT-C plus rrTnI, TnI (L111G), and TnI (K105G) at a molar ratio of 4:6:2.7 of rrTnI: TnT-C:TM:actin and a 9:13:5:2:7 ratio of TnI mutant:TnT-C:TM:actin (the ratios required for maximum Ca2+-dependent change in the ATPase assay, Fig. 2B). The regulatory complex composed of the TnI (L111G) plus TnT-C has a similar pCa50 value as rrTnI plus TnT-C (compare 6.22 to 6.14, respectively, Table I) while a small increase in the pCa value (6.35; Table I) is observed by TnI (K105G) plus TnT-C. This is in agreement with the small increase in Ca2+-sensitivity (0.2 pCa units) when endogenous TnI is substituted with TnI (K105G) in cardiac skinned muscle fiber (20). In addition, skinned fibers reconstituted with TnI (K105G) had a slower rate of fiber relaxation than either fibers reconstituted with rrTnI or unreconstructed fibers which could reflect a decrease in the affinity of this TnI mutant for actin-TM.

**DISCUSSION**

We have outlined a general protocol for the reconstitution of regulatory complexes composed of TnI fragments and mutants which have different affinities for actin-TM, TnT, and/or TnC...
than native TnI. The quantity of the TnI fragment or mutant required for maximum inhibition of the acto-S1-TM ATPase activity is initially determined. At this concentration of inhibitor, increasing quantities of a preformed TnT-C complex is added until maximum Ca\(^{2+}\)-dependent release and/or activation of the ATPase activity occurs. The pCa/ATPase relationship of these regulatory complexes are then assayed at the molar ratio of these proteins required for maximum Ca\(^{2+}\) (similar (even though the concentrations of the regulatory proteins required for maximum Ca\(^{2+}\) are important for binding to both actin-TM and TnC. Third, the inhibitory region can modulate or influence the extent of activation since, a single amino acid substitutions within the inhibitory region of TnI-(96–148) have an important role in the interaction between the acto-S1-TM activity (108 and 119%, respectively). Second, TnT is essential for activation of the ATPase activity since TnI-(104–115) and TnC interaction. This effect was not seen with TnI-(96–148), where TnT-C was very effective at releasing inhibition by TnI-(96–148), reaching maximum ATPase activity (119%) at 2:1 molar ratio of TnT-C:TnI-(96–148) (Fig. 2A). These results indicate that additional residues within TnI-(96–148) have an important role in the interaction between the three subunits of Tn. To date, there has been no report of any interaction between C-terminal regions of TnI and TnT. However, Chandra et al. (14) showed that TnI-(96–116) binds tighter than TnI-(104–115) to TnC while TnI-(96–148) binds even tighter to TnC than either TnI-(96–115) or TnI-(116–148) (15). Taken together, the inhibition and TnT-C release data indicate that residues within TnI-(96–148) other than 104–115 are important for binding to both actin-TM and TnC. On the other hand, the ability of Tn to activate the ATPase activity above the acto-S1-TM ATPase activity resides in the interplay between the N terminus of TnI and TnT. This is shown by several experimental results. First, regulatory complexes reconstituted from rabbit TnT-C plus intact rabbit Tn activates the ATPase activity above acto-S1-TM rates (\(\geq 198\%\) versus 100%) while those formed with the N terminus of TnI deleted (TnI-(104–115) or TnI-(96–148)) had activities close to the acto-S1-TM activity (108 and 119%, respectively). Second, the regulatory complex reconstituted with chicken TnI-(1–116) plus TnT-C activated the ATPase activity to the same degree as rabbit skeletal Tn plus TnT-C. Third, the inhibitory region can modulate or influence the extent of activation since, a single amino acid substitution within the inhibitory region of TnI-(111G) had a significant increase in the level of activation compared with intact TnI (Fig. 2B). Fourth, TnT is essential for activation of the ATPase activity since TnT-C (8, 12) and TnI-(1–116)-C (13) are unable to activate the ATPase activity. Thus, activation (and the degree of activation) of the ATPase activity in the presence of Ca\(^{2+}\) is dependent on the presence of TnT, TnC, the N-terminal region of TnI, and amino acid residues within the TnI inhibitory region (residues 104–115). It has been shown that the N-terminal region of TnI contains

### Table II

| Experiment number | Ratio of components in assay | Mole ratio of protein bound to 7 actin monomers in the pellet |
|------------------|----------------------------|----------------------------------------------------------|
|                  | TM + actin (2:7)           | TnI or fragment TnI TnT TnC TM                           |
| 1                | 1.00 ± 0.01                | 0.74 ± 0.02                                             |
| 2                | 1.00 ± 0.05                | 0.64 ± 0.03                                             |
| 3                | 0.08 ± 0.05                | 0.53 ± 0.05                                             |
| 4                | 1.26 ± 0.01                | 1.40 ± 0.01                                             |
| 5                | 1.18 ± 0.10                | 1.94 ± 0.21                                             |
| 6                | 1.34 ± 0.00                | 1.48 ± 0.07                                             |
| 7                | 1.27 ± 0.06                | 2.08 ± 0.06                                             |
| 8                | 2.02 ± 0.20                | 1.72 ± 0.04                                             |

\(\dagger\) The assayed consisted of 1.5 nmol of actin and 0.428 nmol of TM (dimeric) in an assay volume of 175 \(\mu\)l of binding (100 mm KCl) buffer which contains 3 mm CaCl\(_2\).

\(b\) Data has been corrected for any sedimentation of the various proteins and fragments that occur in the absence of actin.

c Rabbit skeletal TnI.
FIG. 4. The Ca\(^{2+}\) sensitivity of the TnI or TnI fragment reconstituted with TnT-C. The effect of regulatory complexes consisting of TnI or TnI fragments and TnT-C on the acto-S1-TM ATPase was measured as a function of Ca\(^{2+}\) concentration (pCa). The molar ratio of actin:S1:TM was 6:1:2. The average with the standard deviation of two or three independent determinations carried out in triplicate is presented. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point. The results are expressed as a percentage of the Ca\(^{2+}\)-dependent ATPase activity. Panel A shows the effect of various ATPase assay conditions and thin filament reconstitution protocols on the ATPase/pCa relationship compared with native rabbit skeletal Tn (rTn). A 2:2:7 molar ratio of Tn{T}:actin was used with either 200 \(\mu\)l (KCl concentration of 19 mM), or 200 \(\mu\)l assay volume (KCl concentration of 6.5 mM), demonstrating that the salt concentration of the assay buffer does not alter the ATPase/pCa relationship (Table I). A regulatory complex could be reconstituted by renaturing the individual Tn subunits (1:1:1 molar ratio of TnI:TnT:TnC) from urea/KCl with the final dialysis against ATPase (6.5 mM KCl) minus EGTA buffer (pH 7.8). A 2:2:7 molar ratio of TnI-C:TM:actin was used with 200 \(\mu\)l assay volume (final KCl concentration of 6.5 mM, TnT-C, \(\bullet\)). A functional regulatory complex could also be reconstituted by addition of rabbit skeletal TnI and a TnT-C complex (TnI + TnT-C), TnT-C was formed by renaturing skeletal muscle TnT and TnC from urea/KCl according to “Materials and Methods.” A 3:6:7 molar ratio of TnI:TnT:actin was used (the ratios which produced the largest maximum Ca\(^{2+}\)-dependent change) with 200 (final KCl concentration of 19 mM, \(\circ\)) or 320 \(\mu\)l assay volume (final KCl concentration of 13 mM, \(\bigcirc\)). This demonstrates that the assay volume does not affect the results. For panels B-D, the quantity of TnI or TnI fragment and TnT-C was that which caused the maximum Ca\(^{2+}\)-dependent change for each regulatory complex (as determined from Figs. 1 and 2). The ratios of TnI or TnI fragment:TnT-C:actin were 3:6:7, crTnI:3:6:7, TnI-(1–116):3:6:7, TnI-(104–115):3:6:7, TnI-(96–115):3:6:7, TnI-(104–115):6:3:6:7, rrTnI:4:8:7, TnI(K105S):9:13:5:7, TnI(L111G):9:13:5:7. The experiments were carried out at an assay volume of 200 \(\mu\)l (KCl concentration of 19 mM) or 320 \(\mu\)l (KCl concentration of 13 mM), conditions which do not by themselves alter the ATPase/pCa curves (see panel A). Panel B shows the ATPase/pCa relationship for the regulatory complexes composed of TnT-C and the TnI fragments; TnI-(96–148) (\(\bigcirc\)), TnI-(104–115) (\(\bigcirc\)), and TnI-(1–116) (\(\bigcirc\)). Panel C compares the pCa curves for regulatory complexes composed of TnT-C and rrTnI (\(\bullet\)), chicken recombinant skeletal TnI (\(\bigcirc\)), or TnI-(96–148) (\(\bigcirc\)). Panel D shows the ATPase/pCa relationship for the regulatory complexes composed of TnT-C and the TnI mutants with single amino acid residues substituted in the inhibitory region; rrTnI (\(\bigcirc\)), TnI(K105G) (\(\bigcirc\)), or TnI(L111G) (\(\bigcirc\)).

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a TnT-binding site since a skeletal TnI mutant with the first 57 amino acid residues deleted does not bind to TnT (11). The importance of this result is that a troponin regulatory complex composed of this TnI mutant, TnT, and TnC was able to potentiate the acto-S1-TM ATPase activity (12). Thus, the first 57 amino acid residues of TnI which contains the region comprising the regulatory peptide residues 1–40 which binds tightly to TnC (9), are not involved in activation of the ATPase activity. This suggests a region within TnI residues 57–96 interacts with TM, actin, or TnC and is responsible for activation of the ATPase activity.

Ca\(^{2+}\) sensitivity of the reconstituted thin filament is described by the concentration of calcium required to induce 50% of the maximum Ca\(^{2+}\)-dependent change in the ATPase activity (pCa\(_{50}\)). The Ca\(^{2+}\) sensitivity of the reconstituted thin filament is controlled by TnI-(96–148) interaction with TnC. As shown in Fig. 4, the regulatory complex composed of TnI-(96–148) plus TnT-C has the same Ca\(^{2+}\) sensitivity and cooperativity as Tn or intact TnI plus TnT-C while the regulatory complexes reconstituted with TnI-(1–116) or TnI-(104–115) plus TnT-C displayed an increased Ca\(^{2+}\) sensitivity (Fig. 4B). This supports previous results which showed that binding of TnI-(104–115) and TnI-(96–115) to TnC, increases the Ca\(^{2+}\) affinity of the complex compared with TnC alone (14, 35, 36). In addition, S1 is also present in our assay and could effect the Ca\(^{2+}\) affinity of the thin filament (for reviews, see Refs. 5 and 37–39).

The Ca\(^{2+}\) sensitivity may be increased by a reduction in the apparent affinity constant for TnI or TnI fragment with actin-TM or an increase in the apparent affinity constant between TnI or TnI fragment and TnT-C. However, the regulatory complexes in our assays are formed at concentrations of inhibitor and TnT-C that induce maximum inhibition and Ca\(^{2+}\)-dependent change and therefore the composition of the thin filaments are similar for each regulatory complex (Table II). This implies that any effect from the different binding affinities of the various inhibitors for TnT-C or actin-TM should be minimized and the changes in Ca\(^{2+}\) affinity between the various regulatory complexes should reflect a change in association constant of Ca\(^{2+}\) binding to the thin filament.

In summary, distinct regions of TnI regulate the Ca\(^{2+}\)-dependent response of the acto-S1-TM ATPase activity through its interactions with other thin filament proteins. For example, in the absence of Ca\(^{2+}\), TnI residues 96–148 are sufficient for full inhibition of the acto-S1-TM ATPase activity. This data indicates that there must be an additional site of interaction in
the region 96–148 with actin-TM (Fig. 1A) other than the inhibitory site 104–115. It is noteworthy that TnI does not contribute to the maximum level of inhibition of the acto-S1-TM ATPase activity since the TnI fragments, TnI-(96–148) and TnI-(104–115), are able by themselves to inhibit the ATPase activity to the same level as intact TnI or Tn in the absence of Ca2⁺ (Fig. 1). On the other hand in the presence of Ca2⁺, TnI-(104–115) or TnI-(96–148) plus TnTC are responsible for the partial release of the inhibition (to acto-S1-TM ATPase activity). It is the interaction between the N terminus of TnI and TnTC that is responsible for the Ca2⁺-dependent activation of the ATPase activity. In addition, Ca2⁺-sensitivity of the thin filament is controlled through the Ca2⁺-dependent switch of TnI-(96–148) from multiple binding sites on actin to multiple binding sites on TnC(Ca2⁺). Therefore, although the inhibitory region of TnI-(104–115) is the Ca2⁺-sensitive switch (switching binding sites from actin-TM to TnC in the presence of Ca2⁺) its function is modulated by the N and C terminus of TnI.

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Distinct Regions of Troponin I Regulate Ca\textsuperscript{2+}-dependent Activation and Ca\textsuperscript{2+} Sensitivity of the Acto-S1-TM ATPase Activity of the Thin Filament

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