Conformational Modulation of Troponin T by Configuration of the NH_2-Terminal Variable Region and Functional Effects†

Jennifer Wang and Jian-Ping Jin*

Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106-4970

Received May 26, 1998; Revised Manuscript Received July 28, 1998

ABSTRACT: Troponin T (TnT) is an essential element in the thin filament-based regulatory system of striated muscle. Alternative mRNA splicing generates multiple TnT isoforms with primary structural differences in the NH_2-terminal region. The functional significance of this hypervariable NH_2-terminal domain and the developmental or muscle type-specific TnT isoforms is not fully understood. We have analyzed chicken breast muscle TnT containing a metal-binding cluster $[H(E/A)EAH]_{4-7}$ (Tx) in the NH_2-terminal region to demonstrate potential effects of the NH_2-terminal structure on the conformation of TnT [Ogut, O., and Jin, J.-P. (1996) *Biochemistry 35*, 16581−16590]. Using specific antibody epitope analysis on this metal-binding TnT model, this study revealed that the binding of Zn^{2+} to the NH_2-terminal region of chicken breast muscle TnT induces extensive conformational changes in the whole protein as demonstrated by a significant decrease in binding avidity of a polyclonal anti-TnT serum which recognizes multiple epitopes on the TnT molecule. This NH_2-terminal configuration-based effect is not restricted to the metal ion interaction, whereas the binding of anti-NH_2 terminus monoclonal antibodies to TnT induced similar changes. Protein-binding assays have shown that the NH_2-terminal variability-induced conformational changes can alter TnT's binding affinity for tropomyosin and troponin I. The results suggest a functional modulation of TnT through the configuration of the NH_2-terminal domain, and this novel mechanism may mediate the physiological significance of the TnT isoform regulation.

Troponin T (TnT)[1] is the tropomyosin (Tm)-binding subunit of the troponin complex and plays a central role in the thin filament-based regulation of striated (cardiac and skeletal) muscle contraction (*1*−*3*). A large number of TnT isoforms is produced from the transcript of cardiac and slow and fast skeletal muscle TnT genes through developmentally regulated alternative mRNA splicing (*4*−*8*). The primary structure and isoform expression of TnT have been extensively studied in many avian and mammalian species. As the most complex example, the generation of fast skeletal muscle TnT isoforms involves differential splicing of more than six alternative exons (i.e., four to eight, fetal and additional exons found in birds) corresponding to a highly variable NH_2-terminal region and a pair of mutually exclusive exons (16 and 17) encoding a COOH-terminal variable region (*5*, *9*−*12*).

Alternative splicing of an NH_2-terminal acidic segment is responsible for a cardiac TnT isoform switch during both avian and mammalian heart development (*4*, *13*, *14*). Similar to the cardiac TnT isoform switch, a large to small, acidic to basic isoform transition of fast skeletal muscle TnT occurs during skeletal muscle development involving alternative splicing of multiple exons encoding the NH_2-terminal variable region (*12*). Altered cardiac TnT isoform expression has also been observed in various myocardial disorders (*15*, *16*). The functional role of the alternatively spliced variable regions of TnT needs to be established to allow an understanding of the physiological and pathological significance of TnT isoforms.

By studies on rabbit fast skeletal TnT, the protein has been dissected into two major functional domains. The COOH-terminal chymotryptic fragment T2 (Figure 1, residues 159−259) binds to the central region of Tm (*17*, *18*) and interacts with actin, troponin I (TnI), and troponin C (TnC) in a Ca^{2+}-dependent manner (*17*, *19*−*21*). The NH_2-terminal chymotryptic fragment T1 (residues 1−158) has been shown to interact with the COOH terminus of Tm, involving the head-to-tail overlap with the NH_2 terminus of the adjacent Tm molecule (*17*, *18*). While the central region of TnT (fragment CB2 shown in Figure 1, residues 70−150) is required for association of the troponin complex with Tm (*22*), the developmentally regulated NH_2-terminal variable region of

† This study was supported in part by grants from the Medical Research Council of Canada and the Heart and Stroke Foundation of Canada.

* Address correspondence to Dr. J.-P. Jin at Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4970. Telephone: (216) 368-5525. Fax: (216) 368-3952. E-mail: jxj12@po.cwru.edu.

[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; buffer A, 100 mM KCl, 3 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.0); buffer T, buffer A and 0.05% Tween 20; ELISA, enzyme-linked immunosorbant assay; HRP, horseradish peroxidase; mAb, monoclonal antibody; NBT, nitroblue tetrazolium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline [137 mM NaCl, 2.7 mM KH_2PO_4, and 8 mM Na_2HPO_4 (pH 7.4)]; TBS, Tris-buffered saline [150 mM NaCl and 50 mM Tris-HCl (pH 7.5)]; Tm, tropomyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T; Tx, $[H(E/A)EAH]_{4-7}$.

S0006-2960(98)01232-X CCC: $15.00
Published on Web 09/22/1998

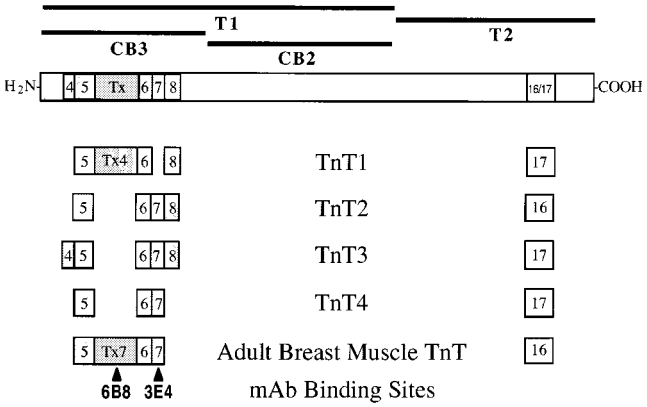

FIGURE 1: Structure of alternatively spliced chicken TnT isoforms used in this study. Regions of the proteolytic T1, T2, CB2, and CB3 fragments of TnT (see the text) are illustrated corresponding to the chicken fast skeletal muscle TnT polypeptide chain. The exon utilization in the alternatively spliced NH_2- and COOH-terminal regions (the boxes in the upper primary structure map) of TnT1−4 as well as a major breast muscle TnT of chicken are outlined. Tx4 and Tx7 indicate the presence of 4 or 7 of the HxxxH metal-binding repeats in TnT1 and the breast muscle TnT, respectively. Locations of the epitopes recognized by mAbs 3E4 and 6B8 are indicated.

TnT (fragment CB3) has not been found to definitively interact with other thin filament proteins (*17*, *19*). Deletion of the first 45 NH_2-terminal residues from rabbit fast skeletal muscle TnT had no significant effect on the Ca^{2+} activation of the thin filament (*22*) and regulation of acto-S1 ATPase (*23*).

In contrast to the results of studies using TnT fragments, functional differences have been observed in studies using intact TnT for the actomyosin ATPase activation by reconstituted thin filaments containing alternatively spliced TnT isoforms with NH_2-terminal primary structure differences (*24*). Scattered point mutations in the cardiac TnT have been found in human familial hypertrophic cardiomyopathy (*25*), suggesting that various subtle changes in TnT structure may cause a class of similar abnormalities in muscle growth and function. On the basis of the finding of a transition metal (Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+})-binding segment ([His-(Glu/Ala)-Glu-Ala-His]$_n$, designated as Tx; *9*) in the NH_2-terminal region of fast skeletal muscle TnT of the avian order Galliformes (*26*), we have previously demonstrated that the metal-induced structural changes in the NH_2-terminal domain can modulate the molecular conformation and Tm interaction of intact TnT (*27*).

To overcome limitations due to a lack of crystallography or NMR structure information for TnT, we have applied monoclonal and polyclonal antibody epitope analysis in monitoring the three-dimensional structure of the NH_2-terminal variable domain and the entire molecule of TnT. Together with the metal−TnT conformational modulation model (*27*), we developed a novel approach for analyzing the structure−function relationships among different domains of TnT. In this study, we have found that the binding of Zn^{2+} or monoclonal antibodies (mAbs) to the NH_2-terminal region of chicken breast muscle TnT induces extensive conformational changes in the entire molecule. The NH_2-terminal domain-mediated secondary conformational changes have significant effects on TnT's interaction with Tm and TnI, an indication of the functional significance of TnT isoforms which differ in the alternatively spliced NH_2-terminal variable region.

MATERIALS AND METHODS

Preparation of TnT Isoforms and Other Thin Filament Proteins Used in This Study

Native Chicken Breast Muscle TnT. TnT was purified from fresh breast muscle of adult white leghorn chickens by a metal-binding affinity chromatography method, which takes advantage of the high-avidity metal-binding Tx element present in the NH_2-terminal domain of the major TnT isoform in adult chicken breast muscle (*26*, *27*). We have demonstrated by cDNA cloning and expression that the adult chicken breast muscle (pectoralis) major fast TnT isoform contains seven of the HxxxH metal-binding sites (*28*) in the NH_2-terminal Tx segment and exon 16 in the COOH-terminal variable region (Figure 1). High-capacity Zn^{2+} affinity chromatography was carried out with a 10 mL Zn^{2+}-charged Chelating Fast Flow Sepharose (Pharmacia) column in phosphate buffer (pH 7.0) containing 1 M NaCl and 6 M urea to achieve a one-step isolation. Sephadex G75 (Pharmacia) gel filtration chromatography was used to purify the protein to homogeneity (*27*).

Cloned Chicken Fast Skeletal Muscle TnT Isoforms. Chicken TnT1−4 isoforms were expressed from cloned cDNAs (*9*) (generously provided by L. B. Smillie, University of Alberta, Edmonton, AB). Generated by alternative mRNA splicing, primary structure configurations of these cloned chicken fast skeletal muscle TnT isoforms as well as the adult breast muscle major TnT are summarized in Figure 1. pET3 or pAED4 expression plasmids encoding these TnT isoforms were constructed as described previously (*29*). Large-scale expression and purification were carried out by transformation of BL21(DE3)pLysS *Escherichia coli* cells. The isopropyl 1-thio-β-D-galactopyranoside-induced bacterial cells were harvested by centrifugation and disrupted with a French press. The TnT isoforms expressed in *E. coli* were present in the soluble fraction after cell lysis, and different procedures were carried out to purify the TnT isoforms according to their chemical and physical properties. The Tx-positive TnT1 isoform expressed in the bacterial culture was purified by the Zn^{2+} affinity method as described above. The bacterially made non-metal-binding chicken TnT isoforms (TnT2−4) were purified by ammonium sulfate precipitation, DE-52 and CM52 ion-exchange chromatography, and G75 gel filtration column fractionation in the presence of 6 M urea (*29*).

Chicken Skeletal Muscle TnI. Fresh adult chicken breast muscle was used for TnI purification by an ion-exchange chromatography method as described previously (*30*).

Chicken α*-Tm.* Frozen chicken hearts (ventricles) were used to purify α-Tm by the heat denaturation enrichment and ion-exchange chromatography method as described previously (*31*).

Biological Activity of the Protein Preparations. The biological activities of muscle or recombinant TnT isoforms and TnI were tested by reconstitution of the troponin complex. As described previously (*29*, *32*), purified chicken TnT, TnI, and TnC in a 1:1:1.4 molar ratio were mixed in a buffer containing 4.6 M urea and 1 M KCl in the presence

of Ca^{2+}. Serial steps of dialysis were carried out to reduce urea to zero and the salt to 6.5 mM KCl. The 40% excess of TnC in the starting mixture was to compensate for its extra loss during dialysis due to its smaller size compared to TnI and TnT. After concentration by Centricon 30 spin filter (Amicron) ultrafiltration, the reconstituted troponin complex was purified by a Sepharose CL-6B gel filtration column from the remaining free subunits.

F-Actin cosedimentation experiments were carried out as described previously (*13*) to verify the biological activity of the purified Tm and troponin. Rabbit skeletal muscle F-actin, chicken α-Tm, and the reconstituted troponin complex in a 7:2:1 molar ratio were incubated in an actin-binding buffer [buffer A, containing 100 mM KCl, 3 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.0)]. The reaction mixtures, together with the separate protein controls, were centrifuged in a Beckman Airfuge to pellet the F-actin and associated proteins. The supernatants and pellets were analyzed by SDS−polyacrylamide gel electrophoresis (PAGE) to examine the reconstitution of thin filament structure by the troponin and Tm preparations.

Preparation of Specific Polyclonal and Monoclonal Antibodies against Chicken TnT

To develop a polyclonal anti-TnT antibody, purified chicken breast muscle TnT was used to immunize a 2 kg male New Zealand white rabbit. The initial immunization was done by intramuscular injection of 1 mg of the immunogen in 500 μL of phosphate-buffered saline [PBS, 137 mM NaCl, 2.7 mM KH_2PO_4, and 8 mM Na_2HPO_4 (pH 7.4)] mixed with an equal volume of Freund's complete adjuvant. At 3 week intervals, the rabbit was boosted three times intramuscularly with 1 mg each of the chicken TnT in Freund's incomplete adjuvant. Serum samples were tested by indirect enzyme-linked immunosorbant assay (ELISA) (*33*), and the rabbit was terminally bled when the anti-TnT antibody titer in the serum rose to 10^{-6}. The antiserum obtained in large quantity was lyophilized in small aliquots for use in various experiments.

A short-term immunization procedure was applied to develop specific anti-TnT mAbs. Eight-week-old female Balb/c mice were injected intraperitoneally and intramuscularly with a total of 500 μg of the purified chicken breast muscle TnT antigen in 100 μL of PBS mixed with an equal volume of Freund's complete adjuvant. Ten days later, the mouse was intraperitoneally boosted daily for three times with 500 μg each of the antigen in 200 μL of PBS without adjuvant on three consecutive days. Two days following the last boost, spleen cells were harvested from the immunized mouse to fuse with SP2/0-Ag14 mouse myeloma cells using 50% polyethaglycol$_{1500}$ (GIBCO/BRL) containing 7.5% dimethyl sulfoxide as described previously (*33*). Hybridomas were selected by HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) media containing 20% fetal bovine serum and screened by indirect ELISA using horseradish peroxidase (HRP)-labeled, goat anti-mouse total immunoglobulin (Sigma) as the second antibody. The anti-TnT antibody-secreting hybridomas were subcloned three or four times by limiting dilution using young Balb/c mouse spleen cells as a feeder to establish stable cell lines. The hybridoma cells were introduced into the 2,6,10,14-tetramethylpentadecane (pristane, Sigma)-primed peritoneal cavity of Balb/c mice to produce mAb-enriched ascites fluids.

Characterization of Anti-Chicken Fast Skeletal Muscle TnT mAbs

Immunoglobulin Subclass Determination. The immunoglobulin subclass of the anti-TnT mAb's was determined by a sandwich ELISA using a mouse immunoglobulin isotyping kit (Pharmingen, San Diego, CA) according to the manufacturer's protocol.

TnT Isoform Specificity. Fresh or frozen skeletal and/or cardiac muscle tissues from nine vertebrate species were homogenized in SDS gel sample buffer containing 1% SDS. As described previously (*12*), the muscle protein extracts were resolved on SDS−PAGE run with the Laemmli buffer system. The protein bands resolved were electrophoretically transferred onto nitrocellulose membrane for Western blotting. The blotted membrane was blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline [TBS, 150 mM NaCl and 50 mM Tris-HCl (pH 7.5)] before being incubated with the anti-TnT mAbs. After alkaline phosphatase-labeled rabbit anti-mouse IgG second antibody (Sigma) reaction and high-stringency washes using TBS containing 0.5% Triton X-100 and 0.05% SDS, chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were used for color development.

Fragment and Epitope Specificity. Western blotting was carried out on the T1 and T2 fragments of chicken breast muscle TnT prepared by limited chymotrypsin digestion (*30*) to identify the NH_2- or COOH-terminal domain specificity of the anti-TnT mAbs. The immunoblotting was performed as above except that the nitrocellulose membrane used had a pore size of 0.22 μm instead of 0.45 μm. The cloned chicken TnT1−4 isoforms with defined structures in the alternatively spliced variable regions (Figure 1) were used in Western blots as described above to characterize the fine epitope specificity of the anti-TnT mAbs.

To examine the relationship between epitopes recognized by the anti-TnT mAbs and the NH_2-terminal Tx element, an indirect ELISA was carried out. Microtiter plates were coated with a 20-mer Tx peptide−BSA conjugate (*26*). After blocking, the plates were incubated with series dilutions (10^{-2}−10^{-5}) of the mAb ascites or a rabbit polyclonal antiserum R207 raised against the 20-mer Tx peptide (*26*). After washes, HRP-conjugated anti-mouse total immunoglobulin or anti-rabbit IgG second antibody (Sigma) was applied followed by H_2O_2−2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate reaction (*27*). The color development was monitored for absorbance at 405 nm by a Bio-Rad Benchmark automated microplate reader.

To verify the location of the mAb 6B8 epitope within the Tx element, a competitive ELISA was performed. As illustrated in Figure 2A, chicken breast muscle TnT at 5 μg/mL was coated on a microtitering plate at 4 °C overnight. A pretitrated concentration of 6B8 antibody was mixed with serial dilutions of the competitive anti-Tx peptide antibody R207 and added to the microtitering plate coated with chicken breast muscle TnT. Following the ELISA procedure described above, HRP-conjugated anti-mouse immunoglobulin second antibody was used to selectively detect the binding of 6B8 mAb. Competition for R207 by mAb 6B8

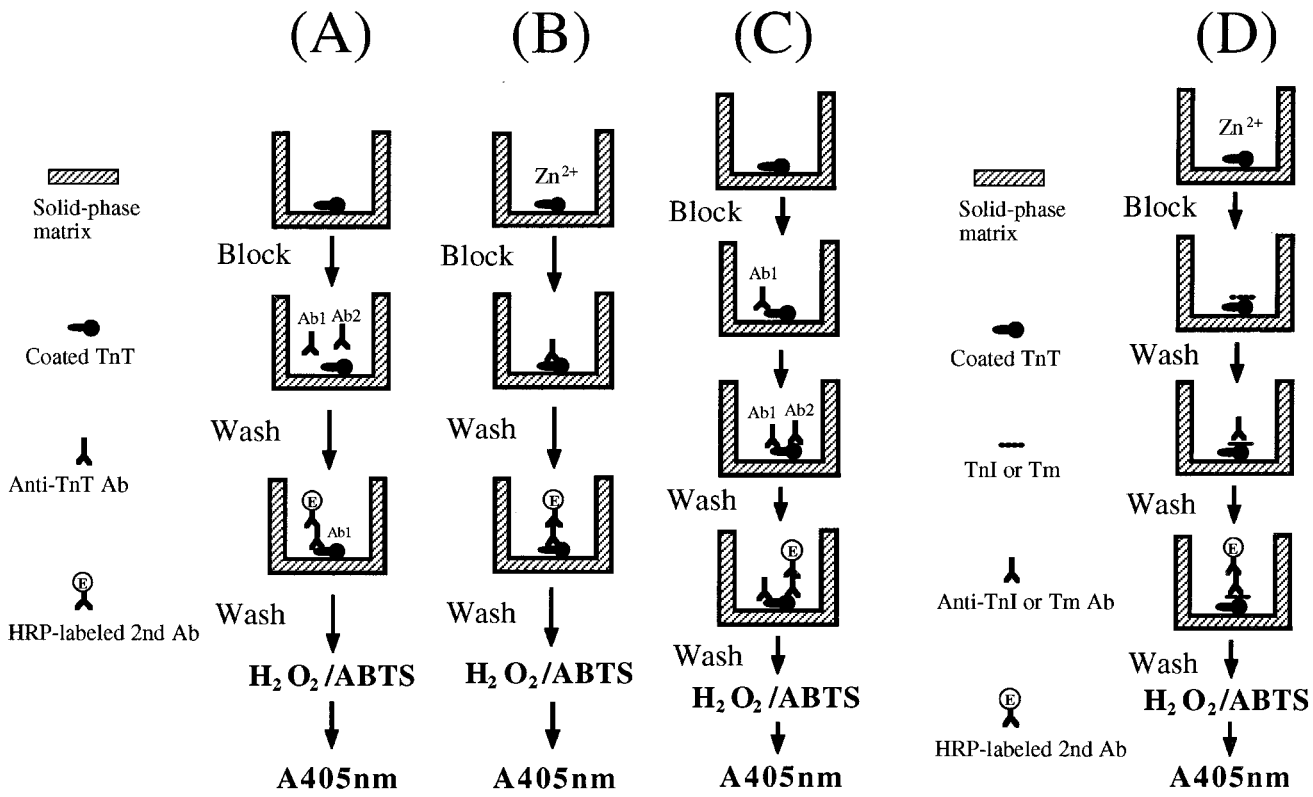

FIGURE 2: ELISA-based epitope analysis and protein binding assays. (A) Competitive ELISA. TnT was coated on a microtitering plate, and an antibody to be tested (Ab1) was added at a pretitrated concentration together with serial dilutions of the competitive antibody (Ab2). HRP-conjugated second antibody specific to Ab1 was used to selectively detect the binding of Ab1 via H_2O_2−ABTS substrate color development. (B) ELISA TnT conformation analysis. TnT is coated on microtitering plates in the presence or absence of Zn^{2+}. After blocking, the wells are incubated with serial dilutions of specific anti-TnT antibodies. Following standard ELISA procedure as described for panel A, the reactivity between the antibody and TnT epitopes is recorded by absorbance at 405 nm. (C) Double-antibody epitope conformational assay. The immobilized TnT is charged with serial dilutions of the anti-NH_2 terminus mouse mAb (Ab1) before adding the rabbit polyclonal antiserum RATnT (Ab2). The extent of binding of the mouse mAb and the rabbit antibody to the coated TnT can be selectively measured via HRP-labeled anti-mouse or anti-rabbit immunoglobulin second antibody, respectively, and substrate reaction as described for panels A and B. (D) Protein binding assay. TnT is coated on microtitering plates in the presence or absence of Zn^{2+}. After blocking, the plates are incubated with serial dilutions of Tm or TnI, followed by anti-Tm or anti-TnI mAb to detect the bound Tm or TnI. The washing, second antibody reaction, and substrate color development are as described for panel A.

was assayed similarly using a pretitrated concentration of R207 and serial dilutions of 6B8 followed by HRP−anti-rabbit immunoglobulin second antibody detection.

ELISA Epitope Analysis for Characterizing Metal-Induced Conformational Changes of TnT

An ELISA-based solid-phase epitope analysis (*27*) was carried out to monitor conformational changes in TnT induced by the binding of metal ions to the Tx segment in the NH_2-terminal variable region. Both primary changes in the Tx element and secondary overall conformational changes induced in the entire TnT molecule were analyzed. As outlined in Figure 2B, purified TnT in buffer A was coated on microtiter plates (Falcon 3915) in the presence or absence of $ZnCl_2$. After the plastic surface was washed and blocked with 1% BSA and 0.05% Tween 20 in buffer A, the immobilized TnT was incubated with serial dilutions of the anti-Tx mAb 6B8 or monoclonal and polyclonal antibodies against epitopes distant from the Tx segment. Following washes with buffer A and 0.05% Tween 20 (buffer T) to remove the unbound antibody, the plates were further incubated with HRP-conjugated second antibody followed by washes and H_2O_2−ABTS substrate reaction. The experiment was carried out in triplicate. The A_{405nm} curve for each assay well was recorded by an automated microplate reader (Bio-Rad Benchmark) to quantify the interaction between the antibody and specific epitopes on the TnT molecule.

ELISA Epitope Analysis of Overall Conformational Changes of TnT Induced by Binding of Anti-NH_2 Terminus mAb

Taking advantage of the availability of anti-TnT specific antibodies raised in different host species, we characterized the effects of NH_2-terminal structural reconfiguration due to the binding of anti-TnT NH_2 terminus mAbs on the conformation of other domains of TnT by a double-antibody ELISA procedure. As illustrated in Figure 2C, chicken breast muscle TnT was coated on microtitering plates (Falcon 3915) and the wells were blocked as described above. The coated TnT was first charged with serial dilutions of the mouse mAb 3E4 or 6B8 in buffer T containing 0.1% BSA for 1 h before a pretitrated dilution of the RATnT rabbit polyclonal antiserum was added. After further incubation for 40 min, the duplicated sets of assay wells were washed with buffer T and then incubated with HRP-conjugated anti-rabbit or anti-mouse immunoglobulin second antibody, respectively, to selectively detect the bound RATnT or 3E4/6B8 mAbs. After final washes with buffer T, the plates were developed with H_2O_2−ABTS substrates and the A_{405nm} was measured as described above in triplicate assays. The relationship

between the extent of binding of mAb to the NH_2 terminus and the TnT's reactivity with respect to the RATnT polyclonal antiserum was plotted to demonstrate the modulation of the TnT overall conformation by the NH_2-terminal structure reconfiguration.

ELISA-Mediated Solid-Phase Protein-Binding Experiments

Similar to the actin-binding assays which we have developed for studying nebulin (*34*) and titin (*35*) fragments, an ELISA-based TnT−Tm binding assay has been adapted (*27*) and modified in this study so the effect of TnT conformational changes on the interaction with Tm and TnI could be characterized. As illustrated in Figure 2D, microtiter plates (Falcon 3915) were coated at 4 °C overnight with purified TnT in buffer A containing 0.1 mM $ZnCl_2$ or 0.1 mM EDTA. After washes to remove the excess TnT and blocking with buffer T containing 1% BSA, the plates were incubated with serial dilutions of chicken α-Tm or breast muscle TnI in buffer A at room temperature for 3.5 h. The plates were then washed with buffer T to remove unbound Tm or TnI before incubation with an anti-Tm mouse mAb CH1 (a gift from J. J.-C. Lin, University of Iowa, Iowa City, IA) (*36*) or an anti-TnI mouse mAb TnI-1 (unpublished results) at room temperature for 1 h. Following washes as above, the HRP-conjugated anti-mouse immunoglobulin second antibody was incubated with the plate at room temperature for 40 min followed by washes and H_2O_2−ABTS substrate color reaction. The A_{405nm} of triplicate assay wells was monitored at a series of time points by a Bio-Rad Benchmark automated microplate reader, and the data within the linear course of the color development were used to plot the TnT−Tm or TnT−TnI binding curves.

RESULTS

Purified TnT Isoforms, Tm, and TnI. Highly purified chicken fast skeletal muscle TnT isoforms, TnI, and cardiac α-Tm were obtained for functional characterization. The native adult chicken breast muscle metal-binding TnT contains mainly the Tx8-e16 isoform as shown by cloning and sequence analysis of a large number of cDNAs (*37*). The expression of cloned TnT isoforms in bacteria provides homogeneous TnT isoforms with defined specific primary structure. The authenticity of the cloned TnT isoforms was verified by their mobility in SDS−PAGE (Figure 3A) and Western blotting using anti-TnT antibodies (Figure 5C). The results showed that the cloned chicken fast skeletal muscle TnT1−4 isoforms have size differences corresponding to their molecular weight deduced from sequence data (*9*) and are all recognized by the specific anti-TnT antibody RATnT. Amino acid analysis was used to determine the protein concentration used in the functional assays, and the demonstrated residue ratio agreed with the sequence-deduced amino acid composition (data not shown). Biological activity of the TnT isoforms as well as the TnI and Tm prepared from muscle by conventional methods or from genetic expression in bacteria has been demonstrated by their ability to reconstitute the troponin complex and the actin thin filament (Figure 3B).

Anti-TnT mAbs with Single-Epitope Specificity and Polyclonal Antiserum Conferring Broad Epitopic Recognition.

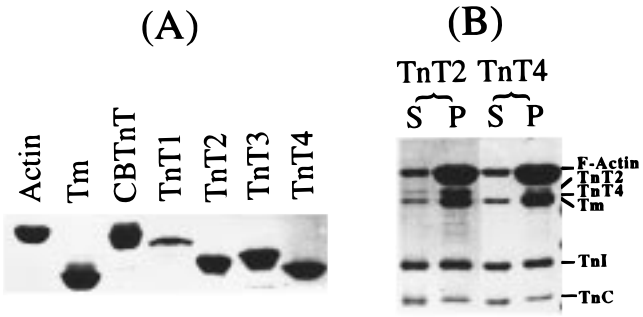

FIGURE 3: Purified TnT isoforms and other thin filament proteins used in this study. (A) The purified rabbit skeletal muscle actin, chicken α-Tm, breast muscle (CB) TnT, and chicken fast skeletal muscle TnT isoforms 1−4 expressed in *E. coli* were analyzed on 14% SDS−PAGE with an acrylamide:bisacrylamide ratio of 37.5:1. (B) SDS−PAGE results of an F-actin cosedimentation assay using the reconstituted troponin complex containing representative TnT isoforms (TnT2 or TnT4), demonstrating that the purified muscle proteins are biologically active. S and P represent supernatant and pellet fractions separated by sedimentation, respectively.

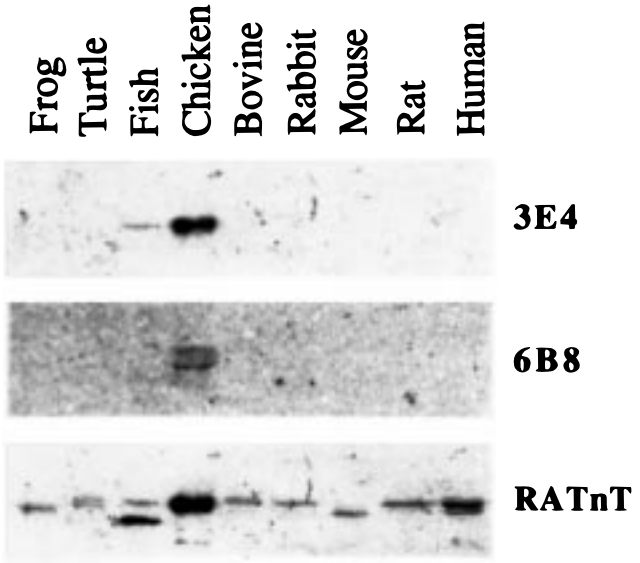

FIGURE 4: Highly specific vs broad reactivity of the monoclonal and polyclonal anti-chicken skeletal muscle TnT antibodies. Total muscle protein extracts from nine vertebrate species were analyzed by SDS−PAGE (12% gel with an acrylamide:bisacrylamide ratio of 29:1) and Western blotting to demonstrate the highly specific vs broad epitope recognition of the monoclonal (3E4 and 6B8) and polyclonal (RATnT) anti-TnT antibodies.

Using the 14 day short-term immunization method and standard hybridoma production procedures, two mouse hybridoma cell lines, 6B8 and 3E4, have been established to secrete high-titer anti-TnT mAbs. Immunoglobulin isotyping determined that the 6B8 mAb is IgG1κ and 3E4, IgG1λ.

A high-titer rabbit anti-TnT polyclonal antiserum was generated by immunization with purified chicken breast muscle TnT. A large quantity of this immunoserum was obtained from a single terminal bleeding. The strategy of obtaining a uniform batch of the polyclonal antibody avoids differences between antisera from repeated bleedings, which often introduce variations in reactivity among experiments.

Tested by Western blotting on protein extracts from skeletal muscles of nine species across the vertebrate phyla, mAbs 3E4 and 6B8 strongly recognize their immunogen,

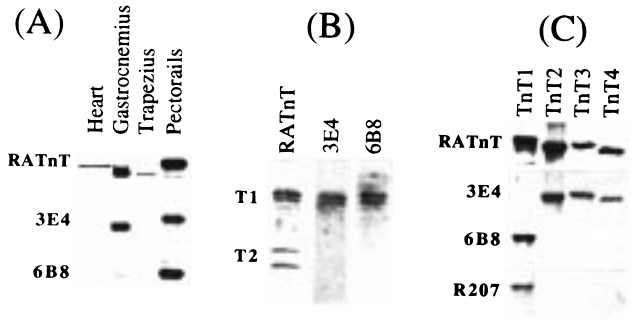

FIGURE 5: Isoform and fragment specificity of the anti-TnT antibodies. Western blotting was carried out as described in the legend of Figure 4. (A) Chicken heart (cardiac muscle), gastrocnemius (containing mainly Tx-negative fast skeletal muscle TnT), trapezius (containing mainly slow skeletal muscle TnT), and pectoralis (containing mainly Tx-positive fast TnT) were used in Western blots to characterize the fast TnT isoform specificity of mAbs 3E4 and 6B8. The RATnT polyclonal antibody was shown to react with all three TnTs expressed in the muscle samples. (B) The chymotryptic fragments of chicken breast muscle TnT were analyzed. In contrast to the broad epitope binding of RATnT polyclonal antibody, an NH_2-terminal specificity of mAb 3E4 and 6B8 was demonstrated. (C) Among the four cloned chicken fast TnT isoforms (TnT1−4, all recognized by the RATnT antibody), the Western blots show that mAb 3E4 recognizes an epitope present in TnT2−4 but not TnT1 which lacks the exon 7-encoded segment, whereas mAb 6B8 recognizes only TnT1 containing the Tx metal-binding segment, similar to the R207 antibody raised against the synthetic Tx_4 peptide (Figure 1).

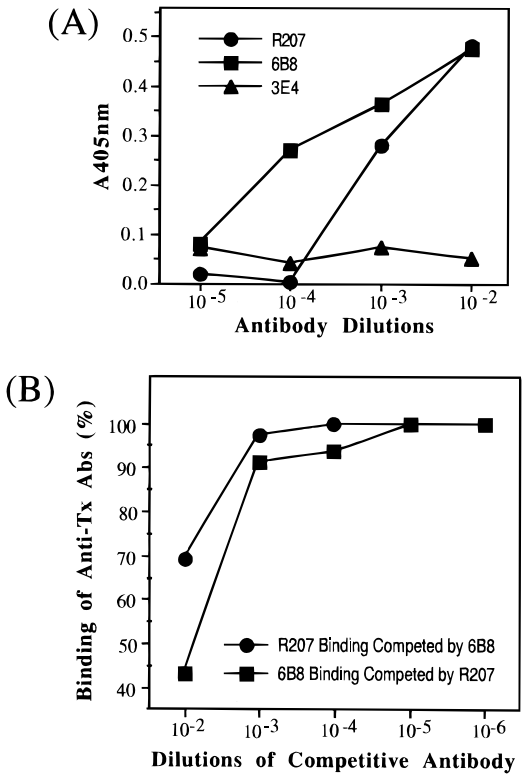

FIGURE 6: Tx specificity of 6B8 mAb. (A) The Tx−BSA conjugate was coated on microtiter plates and reacted with serial dilutions of 6B8 and 3E4 mAb ascites as well as the R207 anti-Tx peptide antiserum. The results show that mAb 6B8 specifically reacts with the Tx−BSA conjugate with an affinity higher than that of R207, whereas mAb 3E4 is negative with respect to the Tx peptide. (B) The results of a competitive ELISA (Figure 2A) show that mAb 6B8 and the anti-Tx peptide antibody R207 compete with each other for overlapping epitope(s) on chicken breast muscle TnT.

chicken breast muscle TnT, with no cross reactivity to frog, turtle, rabbit, bovine, mouse, rat, and human skeletal muscle TnTs. 3E4, but not 6B8, had a weak stain on a minor isoform of the fish TnT (Figure 4). The results indicate that these two mAbs recognize epitopes highly specific for the chicken fast skeletal muscle TnT. In contrast, the polyclonal anti-TnT antiserum showed broad reactivity with respect to skeletal muscle TnTs from all of the species tested (Figure 4), an indication of its recognition of multiple epitopes, including those shared by different vertebrate skeletal muscle TnT isoforms.

The Western blots in Figure 5A show that the polyclonal RATnT antiserum strongly recognizes the chicken fast skeletal muscle TnTs from the gastrocnemius and pectoralis. It also cross reacts with cardiac (from heart) and slow skeletal muscle (from trapezius) TnTs. The reactivity to all three TnTs further demonstrates the broad epitope recognition by this polyclonal antibody. In contrast, the Western blot in Figure 5A demonstrates that without cross reaction with cardiac and slow skeletal muscle TnTs, mAb 3E4 recognizes the pectoralis and gastrocnemius TnT whereas 6B8 reacts only with the pectoralis TnT and a very small portion of the gastrocnemius TnT. Western blots in Figure 5B show that both 6B8 and 3E4 selectively react with the NH_2-terminal T1 fragment of chicken breast muscle TnT while the RATnT polyclonal antibody reacts with both T1 and T2 portions of the molecule. Using the cloned TnT1−4 isoforms with known primary structures (Figure 1), the Western blots in Figure 5C show that mAb 3E4 recognizes TnT2−4 but not TnT1. The collective primary structure difference here is that TnT1 lacks a four-amino acid segment (APPP; *9*) encoded by alternatively spliced exon 7 (Figure 1). Therefore, the mAb 3E4's specific epitope is in the NH_2-terminal variable region of chicken breast muscle TnT. On the other hand, mAb 6B8 which reacts with chicken breast muscle TnT recognizes also TnT1 (both are Tx-positive) with no reaction with the Tx-negative TnT2−4 (Figure 1; *26*), similar to the blotting pattern of the anti-Tx peptide antibody R207 (Figure 5C). The data indicate that the epitope recognized by mAb 6B8 is in the Tx segment which is also within the NH_2-terminal variable region. In contrast, the polyclonal RATnT antiserum strongly recognizes all four cloned chicken fast skeletal muscle TnT isoforms (Figure 5C).

Indirect ELISA on the Tx−BSA conjugate confirmed that mAb 6B8, but not 3E4, recognizes an epitope formed by the 20-mer Tx peptide (Figure 6A), similar to antibody R207 raised against the 20-mer Tx immunogen (*26*). The overlapping relationship between the 6B8 and R207 epitopes was further verified by ELISA competition experiments. Figure 6B shows that mAb 6B8 and the anti-Tx peptide antibody R207 compete with each other for binding to chicken breast muscle TnT. The more effective competition of R207 compared to that of 6B8 agrees with the fact that R207 is a polyclonal antibody which covers potentially multiple epitopes formed by the Tx segment, whereas only a single epitope is recognized by monoclonal 6B8.

Metal−Tx Binding-Induced NH_2-Terminal Structure Reconfiguration and Secondary Effects on the Overall Conformation of TnT. Using the anti-Tx peptide antibody R207, we have previously shown that binding of Zn^{2+} to the Tx element induced a local conformational change within the Tx region (*27*). This allosteric change has been further demonstrated by the metal-induced drop of affinity for mAb

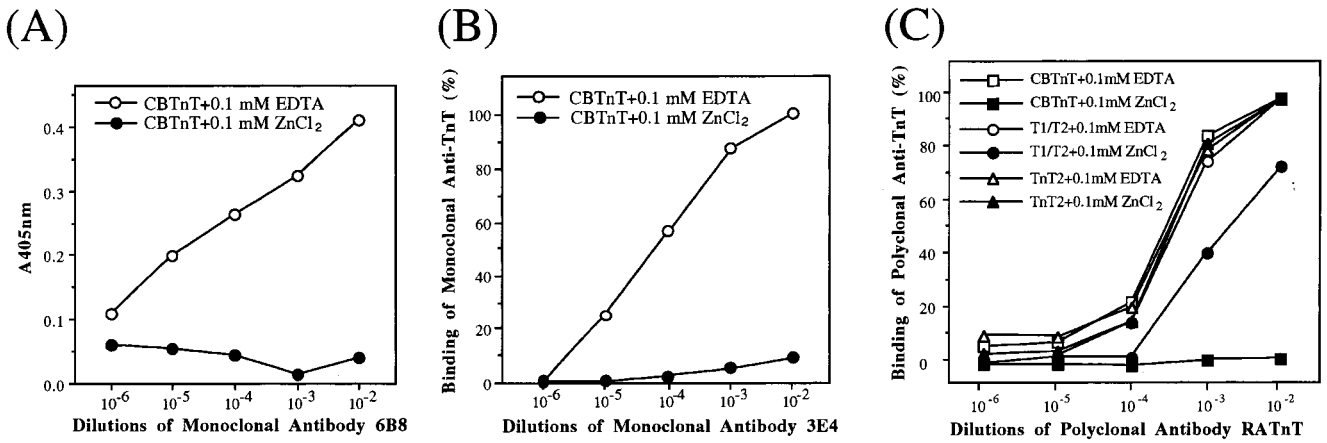

FIGURE 7: Conformational changes in the TnT molecule induced by the binding of Zn^{2+} to the NH_2-terminal Tx segment. ELISA experiments were performed in the presence or absence of Zn^{2+} as illustrated in Figure 2B. (A) The result shows that Zn^{2+} binding changed the local three-dimensional structure of the Tx segment as demonstrated by the dramatic loss of mAb 6B8 binding affinity. (B) The result shows that Zn^{2+} binding to the NH_2-terminal Tx segment had a secondary conformational effect on the downstream exon 7-encoded epitope recognized by mAb 3E4. (C) The result shows that Zn^{2+} binding to the NH_2-terminal Tx segment had extensive conformational effects on the entire TnT molecule as demonstrated by the significant decrease in binding avidity of the polyclonal RATnT antiserum which recognizes multiple epitopes in both NH_2- and COOH-terminal domains of TnT (Figure 5B). In contrast to that of the intact TnT, RATnT's avidity for the mixture of T1 and T2 fragments was only partially affected by the binding of Zn^{2+}, suggesting that only the epitopes on the T1 fragment were reconfigured in their conformation by the binding of Zn^{2+} to the Tx element. The presence of Zn^{2+} had no effect on the reactivity of RATnT with respect to the Tx-negative TnT2.

6B8 which was raised using the native intact TnT antigen. Figure 7A shows that when the Tx-containing chicken breast muscle TnT was pretreated with 0.1 mM $ZnCl_2$, ELISA epitope analysis demonstrated a dramatically decreased extent of binding of mAb 6B8 compared with that in the presence of 0.1 mM EDTA.

Figure 7B demonstrates a remote conformational modulation by the binding of Zn^{2+} to the Tx element. The conformation of the epitope recognized by mAb 3E4 [which is the exon 7-encoded APPP segment downstream of the Tx sequence spaced by five amino acid residues encoded by exon 6 (*9*) (Figure 1)] was significantly affected as a secondary effect of the local conformational change in the Tx segment as shown by the result that Zn^{2+}-treated chicken breast muscle TnT had significantly decreased reactivity with respect to mAb 3E4. This result indicates that Zn^{2+} binding not only reconfigured the Tx structure but also causes secondary conformational changes at a remote site of the TnT molecule.

The results in Figure 7C further demonstrate that the binding of Zn^{2+} to the NH_2-terminal variable region of chicken breast muscle TnT significantly changed the binding avidity of the RATnT polyclonal antibody which recognizes multiple epitopes on TnT. The dramatically diminished reactivity of RATnT indicates that the structure of the TnT molecule was extensively altered as a consequence of the conformational change in the Tx region upon the binding of Zn^{2+}. In contrast, the Tx-negative TnT2's reactivity with respect to RATnT was not affected by the presence of Zn^{2+}, demonstrating that the decreased reactivity of Zn^{2+}-treated chicken breast muscle TnT with respect to RATnT was not due to the nonspecific effect of metal ions on the TnT−antibody interaction.

It is important to note that the pretreatment of T1 and T2 fragments with Zn^{2+} resulted in a partial decrease in the reactivity with respect to RATnT, in contrast to the pronounced loss of reactivity of Zn^{2+}-treated intact TnT with respect to RATnT (Figure 7C). One explanation is that this partial decrease in RATnT binding was due to the conformational change in the T1 region as induced by the Zn^{2+}−Tx interaction. The recognition of epitopes on the T2 region by RATnT remained unchanged in this case since the allosteric effects in the T1 region could not propagate into the T2 fragment due to the disruption of the integrity of the TnT polypeptide chain. The results suggest that interactions between the NH_2- and COOH-terminal domains of TnT in adopting specific molecular conformations depend on the integrity of the protein, instead of on a simple combination of the T1 and T2 domains of the molecule.

Effect of the Binding of Anti-NH_2 Terminus mAb on the Overall Conformation of TnT. To characterize the role of NH_2-terminal configuration in the modulation of TnT molecular conformation, binding of anti-NH_2 terminus mAbs to TnT was shown to result in significant changes in the binding avidity of the polyclonal RATnT antibody (Figure 8). The binding curves shown in Figure 8 reveal that the more 6B8 or 3E4 mAb bound to TnT (detected via the anti-mouse immunoglobulin second antibody), the lower the RATnT's reactivity with respect to TnT (detected via the anti-rabbit immunoglobulin second antibody). The similar secondary effects induced by the interactions of Zn^{2+} or a protein (mAb) with the NH_2 terminus are an indication of a common mechanism by which the structural configuration of the NH_2-terminal variable region can modulate the overall conformation of the TnT molecule. The dose−response curves for the two anti-NH_2 terminus mAbs and the RATnT polyclonal antiserum were not linear inverse relationships. A lag is present between the binding of mAb to the NH_2 terminus and the decrease in RATnT binding (Figure 8). This kinetic feature supports the idea that the change in RATnT binding affinity represents a secondary conformational change in the TnT molecule rather than a simple steric blocking effect caused by binding of an mAb to the NH_2 terminus.

Effect of Zn^{2+} Binding on the Interaction of Tx-Positive TnT with Tm and TnI. As demonstrated by the ELISA-

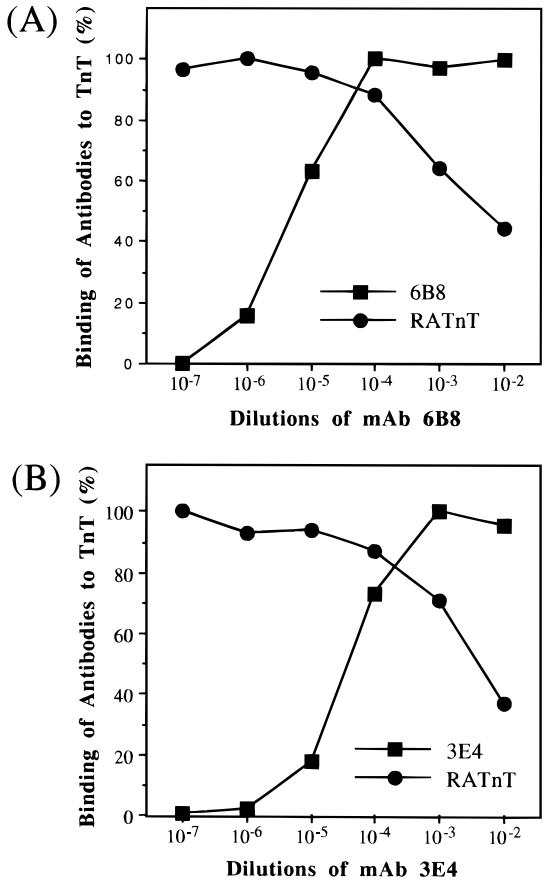

FIGURE 8: Effect of binding of mAb 6B8 or 3E4 to the NH_2 terminus on the overall conformation of TnT. Double-antibody ELISA epitope analysis was carried out as illustrated in Figure 2C to investigate the effects of 3E4 or 6B8 mAb binding to the NH_2-terminal variable region on the overall conformation of chicken breast muscle TnT. The results show an inverse relationship between the binding of anti-NH_2 terminus mAbs and the RATnT polyclonal antiserum against multiple epitopes on the TnT molecule, indicating that this protein−protein interaction-based reconfiguration of TnT NH_2-terminal structure induced secondary overall conformational changes in the protein.

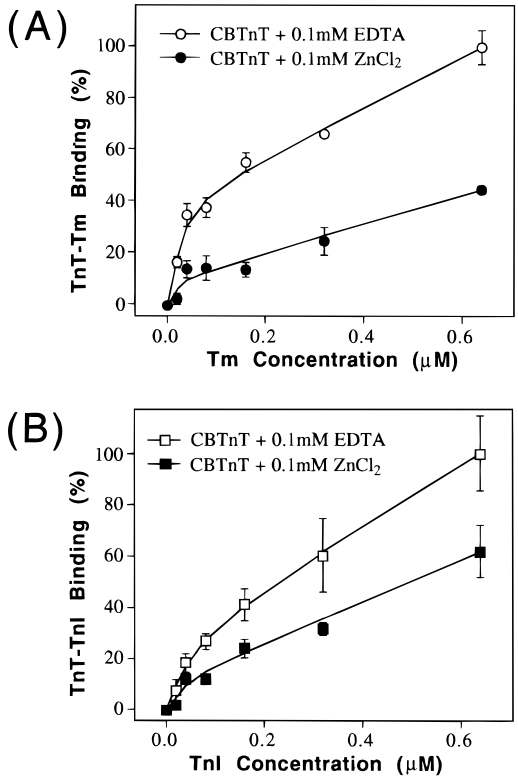

FIGURE 9: Effect of Zn^{2+} on the binding affinity of chicken breast muscle TnT for Tm and TnI. (A) As illustrated in Figure 2D, chicken breast muscle TnT was tested in the ELISA solid-phase protein binding experiment for interaction with Tm in the presence or absence of 0.1 mM $ZnCl_2$. Detected via the anti-Tm mAb CH1, the bound Tm was plotted as the percentage of the maximum level of binding against the concentration of added total Tm (calculated on the basis of the Mr of Tm monomer) to show a significantly decreased binding affinity of TnT as induced by the Zn^{2+} binding to the NH_2-terminal Tx sties ($P < 0.01$). (B) Similar to the TnT−Tm binding assay shown in panel A, the effect of Zn^{2+} binding to the NH_2 terminus of chicken breast muscle TnT on the TnT−TnI interaction was examined by the ELISA method via an anti-TnI mAb. A significantly decreased affinity of TnT for TnI was observed in the presence of 0.1 mM $ZnCl_2$ ($P < 0.05$). The binding curves were constructed using Igor software from Wavemetrics, Inc. One-way analysis of variance (ANOVA) was applied for the statistic comparison.

mediated solid-phase protein binding experiments, treating the Tx-positive chicken breast muscle TnT with $ZnCl_2$ caused a dramatic decrease in the TnT−Tm binding affinity (Figure 9A). This result shows that charging chicken breast muscle TnT with Zn^{2+} at the NH_2-terminal Tx site generated a significant decrease in its reactivity with respect to Tm, although the Tx region per se does not directly interact with Tm. On the basis of the Zn^{2+}-induced overall conformational change as demonstrated in Figure 7C, the change in Tm binding affinity is caused by secondary conformational changes in other domains of TnT. The ELISA protein binding experiment also showed that the treatment of chicken breast muscle TnT with Zn^{2+} caused a significant decrease in TnT's binding affinity for TnI (Figure 9B), further demonstrating that a local structural change in the Tx segment is able to affect not only the overall conformation but also the functions of TnT. Compared to the effect on TnT−Tm interaction (the concentration of Tm required for 50% of the maximum level of binding shifted from 0.13 to >0.64 μM), the Zn^{2+}−Tx binding-induced change of TnI binding affinity was not as dramatic (the concentration of TnI required for 50% of the maximum level of binding shifted from 0.22 to 0.47 μM). The separate effects suggest that different molecular structures are involved in the two most important functional interactions of TnT, which are modulated differentially in response to altered configurations of the NH_2-terminal variable region.

DISCUSSION

mAbs Recognizing Specific Epitopes in the Alternatively Spliced NH_2-Terminal Variable Region of Fast Skeletal Muscle TnT. In this study, we have developed two specific anti-TnT mAbs, 3E4 and 6B8. mAbs produced by hybridoma technology provide homogeneous reagents specific to a unique epitope on a protein (*38*). Epitope mapping experiments have demonstrated that 3E4 recognizes a proline cluster (APPP) encoded by exon 7 (Figures 1 and 5C), whereas 6B8 recognizes the Tx metal-binding cluster containing 7 (in the breast muscle TnT, Figures 4 and 5) or 4 (in TnT1 and the Tx−BSA conjugate, Figures 5 and 6) of the HxxxH sequence repeats encoded by exon X. The NH_2-terminal specificity of the two anti-TnT mAbs (Figure 1) provides novel tools for site-specific modulation as well as

for detection of the structure−conformation relationship of TnT.

The fact that we obtained two anti-NH_2 terminus mAbs following immunization using intact native chicken breast muscle TnT suggests a strong immunogenicity of the NH_2-terminal domain which may be highly exposed in the isolated TnT molecule. This supports the current model in which TnT has an extended structure (*39*). The fact that the Tx structure can readily generate specific antibody immune responses also suggests that the epitopic structure on the NH_2-terminal domain of TnT can act in isolation from other regions of the molecule. In separate experiments, we have demonstrated that the structure of the Tx epitope is independent of the flanking polypeptides as it can be constructed at the very NH_2 terminus of a fusion protein while retaining its original affinity for 6B8 mAb (data not shown).

Antibody Epitope Analysis for Monitoring Protein Conformational Changes. By broad definition, antigen−antibody recognition is a specific protein−protein binding interaction. Like other protein−protein interactions, it depends on a conformational fitting between the antigenic epitope and the antibody paratope, both of which are formed by protein three-dimensional structures. mAbs provide reagents for recognizing a single antigenic determinant, i.e., a specific epitope. Therefore, the use of mAbs or monospecific antibodies provides a novel approach for monitoring protein conformation, structural dynamics, and folding (*40*). In contrast to mAb's recognition of a single epitope, a polyclonal antibody recognizes multiple epitopes on the antigen molecule and provides a useful tool for detecting overall conformational changes of the protein. By the ELISA-mediated antibody epitope analysis, we have demonstrated in a study of smooth muscle calponin point mutations that the conformational changes produced by a single Ser to Asp or Thr substitution can be detected by a battery of anti-calponin mAbs (*41*). Therefore, due to the high-affinity-dependent nature of the solid-phase ELISA method, this epitope analysis is sufficiently sensitive to detect even minor changes in the fitting between the antigen epitope and the antibody paratope. For proteins such as TnT, whose crystal or NMR three-dimensional structure is not yet available, the antibody epitope assay which we have developed provides a valuable approach to quantitatively monitoring conformational changes of the protein under various conditions.

Metal-Induced Structural Changes in the Tx Segment and Secondary Modulation of the Conformation of TnT. Since molecular integrity may be an important factor in TnT's structure and function, studies in which TnT fragments or truncated proteins are used may not be able to provide complete information about the functional significance of the alternatively spliced NH_2-terminal region. Therefore, experiments characterizing intact TnT isoforms are likely to be physiologically more relevant and may be able to show subtle differences in terms of their roles in regulating the striated muscle thin filament. The binding of Zn^{2+} to the Tx sites alters the three-dimensional structure of the Tx segment (Figure 7A). This original structural reconfiguration further affects the conformation of epitopes outside the Tx element as demonstrated by the change in the binding affinity of mAb 3E4 (Figure 7B) which recognizes a downstream epitope (Figure 1). This observation indicates that the structural reconfiguration within the Tx region is propagated into a remote epitopic structure. This intramolecular modulation has been further demonstrated by the result that Zn^{2+} binding to the NH_2-terminal Tx segment of chicken breast muscle TnT significantly altered the binding affinity of polyclonal antibody RATnT against multiple epitopes throughout the TnT molecule (Figure 7C), inferring an extensive secondary conformational change.

The NH_2 terminus-based conformational modulation of the TnT molecule is dependent on the peptide chain linkage between the T1 and T2 domains, i.e., the integrity of the protein, as shown by the conservation of partial immunoreactivity of the T1/T2 mixture with respect to RATnT in the presence of Zn^{2+} (Figure 7C). In this case, the NH_2 terminus-originating conformational information within the T1 fragment was not able to be transmitted into the T2 region, whereas epitopes on the T2 fragment largely retained their binding affinity for the RATnT antibody. This observation also supports a hypothesis in which the alternatively spliced NH_2-terminal variable region of TnT has evolved as an enhancing or modulatory structure. Therefore, its removal from the polypeptide chain would not cause a loss of the basic activity of TnT (*23*, *42*), whereas when it is present as structural variants in intact TnT isoforms, it will confer active function by modulating molecular conformation.

Alteration of the Overall Conformation of TnT as a Result of the Binding of Anti-NH_2 Terminus mAb. When anti-NH_2 terminus mAb 6B8 or 3E4 was applied to introduce a reconfiguration of the hypervariable region, the overall conformation of the TnT molecule was significantly altered. Binding of the anti-NH_2 terminus mAb reduced the affinity of RATnT polyclonal antiserum for multiple epitopes on TnT (Figure 8). This result indicates that not only the binding of metal ions to the NH_2 terminus, but also structural changes resulting from binding of a protein to this region can alter the overall conformation of TnT, confirming that the structural information contained in the NH_2-terminal hypervariable region may be passed along the molecule to modulate the biological function of TnT. This novel observation further supports the hypothesis that it is the NH_2-terminal domain configuration, rather than an effect of specific metal ions on the Tx-positive chicken TnT, that modulates the overall conformation of TnT isoforms.

Modulation of the Interaction of TnT with Tm and TnI through Reconfiguration of the NH_2-Terminal Domain. A change in epitope binding affinity for specific antibodies may reflect a change in the static conformation of the antigen as well as a change in molecular flexibility, and both may result in changes in function of the protein. The finding that the NH_2-terminal structure-induced conformational changes have significant effects on TnT's binding affinity for Tm and TnI (Figure 9) indicates a functional modulatory role of TnT isoforms with primary structure differences in the alternatively spliced NH_2-terminal variable region. Remote structure−function modulation has also been observed in the Tm molecule wherein chemical modification of Tm at Cys-190 had inhibitory effects on head-to-tail polymerization, indicating that structural reconfiguration at Cys-190 can affect the structure and function of the COOH terminus which is 94 residues away in the rod-shaped protein (*2*). Therefore, this may be a common mechanism which confers the highly cooperative action of muscle proteins during contraction. This mechanism may also inderlie the physiological significance

of the developmental and pathological TnT isoform regulations and contribute to the etiopathological effect of TnT NH_2-terminal point mutations which cause human familial hypertrophic cardiomyopathies (*43*). Detailed mechanisms for this molecular structure modulation remain to be established. X-ray crystallography and NMR structural information are critical for further investigation of the functional aspects of the TnT conformational changes demonstrated in this study and for development of an integrated and comprehensive hypothesis for structure−function relationships for TnT and the alternatively spliced NH_2-terminal hypervariable region.

ACKNOWLEDGMENT

We thank Gail McMartin for technical assistance in the production of mAbs, Dr. Jim Lin for the CH1 mAb, and Dr. Larry Smillie for invaluable material support and encouragement.

REFERENCES

1. Leavis, P. C., and Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235−305.
2. Zot, A. S., and Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535−559.
3. Tobacman, L. S. (1996) *Annu. Rev. Physiol. 58*, 447−481.
4. Cooper, T. A., and Ordahl, C. P. (1985) *J. Biol. Chem. 260*, 11140−11148.
5. Breitbart, R. E., and Nadal-Ginard, B. (1986) *J. Mol. Biol. 188*, 313−324.
6. Gahlmann, R., Troutt, A. B., Wade, R. P., Gunning, P., and Kedes, L. (1987) *J. Biol. Chem. 262*, 16122−16126.
7. Jin, J.-P., Huang, Q.-Q., Yeh, H. I., and Lin, J. J.-C. (1992) *J. Mol. Biol. 227*, 1269−1276.
8. Jin, J.-P., Chen, A., and Huang, Q.-Q. (1998) *Gene 214*, 121−129.
9. Smillie, L. B., Golosinska, K., and Reinach, F. C. (1988) *J. Biol. Chem. 263*, 18816−18820.
10. Briggs, M. M., and Schachat, F. (1993) *Dev. Biol. 158*, 503−509.
11. Schachat, F., Schmidt, J. M., Maready, M., and Briggs, M. M. (1995) *Dev. Biol. 171*, 233−239.
12. Wang, J., and Jin, J.-P. (1997) *Gene 193*, 105−114.
13. Jin, J.-P., and Lin, J. J.-C. (1988) *J. Biol. Chem. 263*, 7309−7315.
14. Jin, J.-P., Wang, J., and Zhang, J. (1996) *Gene 168*, 217−221.
15. Akella, A. B., Ding, X. L., Cheng, R., and Gulati, J. (1995) *Circ. Res. 76*, 600−606.
16. Saba, Z., Nassar, R., Ungerleider, R. M., Oakeley, A. E., and Anderson, P. A. W. (1996) *Circulation 94*, 472−476.
17. Pearlstone, J. R., and Smillie, L. B. (1982) *J. Biol. Chem. 257*, 10587−10592.
18. Heeley, D. H., Golosinska, K., and Smillie, L. B. (1987) *J. Biol. Chem. 262*, 9971−9978.
19. Pearlstone, J. R., and Smillie, L. B. (1985) *Can. J. Biochem. 63*, 212−218.
20. Schaertl, S., Lehman, S. S., and Geeves, M. A. (1995) *Biochemistry 34*, 15890−15894.
21. Jha, P. K., Leavis, P. C., and Sarkar, S. (1996) *Biochemistry 35*, 16573−16580.
22. Fisher, D., Wang, G., and Tobacman, L. S. (1995) *J. Biol. Chem. 270*, 25455−25460.
23. Pan, B.-S., Gordon, A. M., and Potter, J. D. (1991) *J. Biol. Chem. 266*, 12432−12438.
24. Tobacman, L. S., and Lee, R. (1987) *J. Biol. Chem. 262*, 4059−4064.
25. Watkins, H., McKenna, W. J., Thierfelder, L., Suk, H. J., Anan, R., O'Donoghue, A., Spirito, P., Matsumori, A., Moravec, C. S., Seidman, J. G., and Seidman, C. E. (1995) *N. Engl. J. Med. 332*, 1058−1064.
26. Jin, J.-P., and Smillie, L. B. (1994) *FEBS Lett. 341*, 135−140.
27. Ogut, O., and Jin, J.-P. (1996) *Biochemistry 35*, 16581−16590.
28. Arnold, F. H., and Haymore, B. L. (1991) *Science 252*, 1796−1797.
29. Jin, J.-P., Ferro, J., Pearlstone, J. R., Fujimori, K., Reinach, F., and Smillie, L. B. (1991) *J. Cell Biol. 115*, 180a.
30. Mak, A. S., Golosinska, K., and Smillie, L. B. (1983) *J. Biol. Chem. 258*, 14330−14334.
31. Smillie, L. B. (1982) *Methods Enzymol. 85*, 234−241.
32. Chandra, M., da Silva, E. F., Sorenson, M. M., Ferro, J. A., Pearlstone, J. R., Nash, B. E., Borgford, T., Kay, C. M., and Smillie, L. B. (1994) *J. Biol. Chem. 269*, 14988−14994.
33. Jin, J.-P., Walsh, M. P., Resek, M. E., and McMartin, G. (1996) *Biochem. Cell Biol. 74*, 187−196.
34. Jin, J.-P., and Wang, K. (1991) *J. Biol. Chem. 266*, 21215−21223.
35. Jin, J.-P. (1995) *J. Biol. Chem. 270*, 6908−6916.
36. Lin, J. J.-C., Chou, C.-S., and Lin, J. L.-C. (1985) *Hybridoma 4*, 223−242.
37. Ogut, O., and Jin, J.-P. (1998) *J. Biol. Chem.* (in press).
38. Köhler, G., and Milstein, C. (1975) *Nature 256*, 495−497.
39. Flicker, P. F., Phillips, J. G. N., and Cohen, C. (1982) *J. Mol. Biol. 162*, 495−501.
40. Goldberg, M. E. (1991) *Trends Biochem. Sci. 16*, 358−362.
41. Tang, D.-C., Kang, H.-M., Jin, J.-P., Fraser, E. D., and Walsh, M. P. (1996) *J. Biol. Chem. 271*, 8605−8611.
42. Fujita, S., Maéda, K., and Maéda, Y. (1992) *J. Biochem. 112*, 306−308.
43. Lin, D., Babkova, A., Homsher, E., and Tobacman, L. S. (1996) *J. Clin. Invest. 97*, 2842−2848.

BI9812322