Expression, Zinc-Affinity Purification, and Characterization of a Novel Metal-Binding Cluster in Troponin T: Metal-Stabilized α-Helical Structure and Effects of the NH_2-Terminal Variable Region on the Conformation of Intact Troponin T and Its Association with Tropomyosin[†]

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ABSTRACT: A repeating metal-binding ($Cu^{2+} > Ni^{2+} > Zn^{2+} \approx Co^{2+}$) sequence $[HE/AEAH]_4$ (Tx) has been recently identified in the NH_2-terminal variable region of troponin T (TnT) isoforms specifically expressed in the breast but not leg muscles of the avian orders of *Galliformes* and *Craciformes* [Jin, J.-P., & Smillie, L. B. (1994) *FEBS Lett. 341*, 135−140]. In the present study, two expression plasmids were constructed to produce chicken TnT1 NH_2-terminal fragments of 47 (N47) or 165 (N165) amino acids containing the Tx metal-binding cluster. The recombinant protein/peptide was expressed in *Escherichia coli* BL21(DE3)pLysS and purified by a highly effective Zn^{2+}-affinity chromatography method. Amino acid analyses, NH_2-terminal peptide sequencing, mass spectrometry and immunological identification confirmed the authenticity of the genetically engineered TnT fragments. In the presence of 2,2,2-trifluoroethanol, transition metals had significant effects on the secondary structure of TnT fragment N47, as shown by circular dichroism. N165 in non-denaturing buffer demonstrated α-helical content comparable to previous data from rabbit fast skeletal TnT fragment T1. Zn^{2+}-binding avidity of the metal-binding TnT and its fragments demonstrated tertiary relationships between the NH_2-terminal variable region and the COOH-terminal segment of the intact TnT protein. Solid-phase protein-binding assays established that Zn^{2+}-binding to the Tx cluster induces epitopic structure changes in this NH_2-terminal segment, further affecting other epitopic structures of intact TnT as well as the function of TnT's tropomyosin binding-sites. The results demonstrate that metal ion-binding to the Tx cluster reconfigures the overall conformation of TnT through structural relationships between the NH_2-terminal variable region and other domains of the intact TnT molecule. Accordingly, the developmental and/or muscle type specific NH_2-terminal structure of TnT isoforms may modulate the Ca^{2+}-activation of muscle contraction.

Troponin T (TnT)[1] is the tropomyosin-binding subunit of the troponin complex and participates in the Ca^{2+}-dependent regulation of contraction of vertebrate striated muscles (Leavis & Gergely, 1984). The cardiac and skeletal muscle TnTs are encoded by different genes and muscle type specific or developmentally regulated isoforms are expressed from each gene through alternative mRNA splicing (Cooper & Ordahl, 1985; Breitbart & Nadal-Ginard, 1986; Jin *et al*., 1992). In fast skeletal muscle TnT, alternative mRNA splicing involving a pair of mutually exclusive COOH-terminal exons (16/17) and seven (Breitbart & Nadal-Ginard, 1986; Smillie *et al*., 1988; Briggs & Schachat, 1993) or possibly more (Schachat *et al.*, 1995) exons encoding a variable NH_2-terminal region produces a large number of protein isoforms. Although the functional significance of TnT isoform diversity is not fully understood, certain pathological conditions have demonstrated a concurrent change in TnT isoform expression (Gulati *et al*., 1994; Akella *et al*., 1995; Anderson *et al.*, 1995). Furthermore, a relationship between cardiac TnT mutation and human familial cardiomyopathies has been reported (Thierfelder *et al*., 1994; Watkins *et al.*, 1995). Although these findings document TnT's importance in the contractile apparatus, studies on the structure and function of intact TnT have been limited, in part due to its low solubility in physiological buffers. In this respect, previous work on rabbit fast skeletal TnT had dissected the protein into two major functional domains. The COOH-terminal chymotryptic fragment T2 (residues 159−259) binds to the central region of tropomyosin (Tm) (Ohtsuki, 1979; Morris & Lehrer, 1984) as well as interacting with actin, troponin I and troponin C (Pearlstone & Smillie, 1978, 1980, 1982; Heeley & Smillie, 1988; Schaertl *et al.*, 1995). The NH_2-terminal chymotryptic

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[1] Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid); BCIP, 5-bromo-4-chloro-3-indolylphosphate; BSA, bovine serum albumin; Buffer A, 0.1 M KCl, 10 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$; Buffer T, Buffer A + 0.05% Tween-20; CD, circular dichroism; ELISA, enzyme-linked immunosorbant assay; IPTG, isopropyl-1-thio-β-D-galactopyranoside; mAb, monoclonal antibody; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4); PBS-T, PBS + 0.05% Tween-20; PCR, polymerase chain reaction; TBS, Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.5); TFE, 2,2,2-trifluoroethanol; Tm, tropomyosin; TnT, troponin T; Tx, $[HE/AEAH]_4$.

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fragment T1 (residues 1−158) has been shown to interact with the carboxy terminus of Tm, extending to include the amino terminus of the adjacent Tm molecule in the head to tail overlap of Tm along the thin filament (Pato *et al*., 1981; Brisson *et al*., 1986; White *et al*., 1987). The central region of TnT (residues 70−150) has been shown to contribute to the tight association of the troponin complex to Tm (Fisher *et al*., 1995). In contrast, the variable NH_2-terminal region of TnT has not been directly associated with a defined function (Pearlstone & Smillie, 1982). Although a TnT isoform switch, primarily due to alternative splicing of this NH_2-terminal region, is well regulated during avian and mammalian heart development (Cooper & Ordahl, 1985; Jin & Lin, 1988; Jin *et al*., 1990), deletion of the first 45 NH_2-terminal residues from rabbit fast skeletal TnT was shown not to affect the cooperative response of regulated actoS1 ATPase to Ca^{2+} (Pan *et al*., 1991). On the other hand, differences in the Ca^{2+}-sensitivity of actomyosin ATPase have been observed in reconstituted systems containing two bovine cardiac TnT isoforms differing in the NH_2-terminal variable region (Tobacman & Lee, 1987). Interestingly, this NH_2-terminal region may contain structures independent of TnT's function in the regulation of contraction. Protein (Wilkinson *et al*., 1984) and cDNA (Smillie *et al*., 1988) sequencing of the chicken breast muscle fast TnT have revealed an unusual 20 amino acid stretch consisting of four regularly repeated histidine pairs $[HE/AEAH]_4$ found within the NH_2-terminal variable region. Recently, this segment (designated Tx) has been shown to be exclusive to TnT isoforms expressed only in the avian orders of *Galliformes* and *Craciformes* (Jin & Smillie, 1994). The Tx segment is able to bind with high affinity to the transition metal ions $Cu^{2+} > Ni^{2+} > Zn^{2+} \approx Co^{2+}$ (Jin & Smillie, 1994), potentially through the four histidine pairs arranged in an α-helical conformation (Arnold & Haymore, 1991; Arnold, 1991). It has been suggested that the number of metal-binding sites made available by Tx are adequate to affect the concentrations of metal ions in the muscle cells (Jin & Smillie, 1994). The importance of essential metals such as Zn^{2+} in intracellular processes has been demonstrated in an increasing volume of experimental evidence, especially in the control of development and differentiation (Nusslein-Volhard *et al*., 1987; Wieschaus & Nusslein-Volhard, 1980).

To further study this novel metal-binding cluster and its effects on the conformation and function of TnT, we have constructed expression plasmids encoding chicken breast muscle TnT1 NH_2-terminal fragments of 47 and 165 amino acids, both incorporating the Tx segment. Having achieved high level expression in *E. coli* and facilitated purification by metal-affinity chromatography, we show that the secondary and tertiary structure of the Tx segment is reconfigured by specified transition metal ions. The binding of Zn^{2+} to the NH_2-terminal Tx element is shown to induce changes in the conformation of other domains of TnT, affecting TnT's interaction with Tm. Demonstrating novel structural relationships between the NH_2-terminal region and other domains in an intact TnT molecule, we provide evidence that the structure of the variable NH_2-terminus of different TnT isoforms may adjust the overall conformation of TnT and modulate the regulation of muscle contraction.

MATERIALS AND METHODS

The core recombinant DNA and protein chemistry techniques used can be found in *Current Protocols in Molecular Biology* (Ausubel *et al*., 1995), *Molecular Cloning: A Laboratory Manual* (Sambrook *et al*., 1989), or as described previously (Jin, 1995).

Construction of Expression Plasmids Encoding NH_2-Terminal Fragments of Chicken TnT1

To prepare the nonfusion TnT NH_2-terminal fragment proteins used in this study, two recombinant plasmids (pTx47 and pTx165) were constructed using the T7 RNA polymerase-based (Studier *et al*., 1990) pAED4 expression vector for protein expression in *E. coli*. Using synthetic oligonucleotide primers and a recombinant pAED4 expression plasmid encoding the intact chicken breast TnT1 isoform (Smillie *et al*., 1988; Jin *et al*., 1991) as the template, two cDNA fragments encoding NH_2-terminal 47 (N47) or 165 (N165) amino acids were amplified by polymerase chain reaction (PCR). The N47 DNA fragment was double-digested with *Xba*I and *Eco*RI, and ligated to a similarly digested pAED4 vector. The N165 DNA fragment was digested with *Xba*I on the 5′ end and ligated to the *Xba*I site of *Xba*I/*Eco*RI digested pAED4 vector DNA. The non-complementary ends of the recombinant plasmid DNA were rendered blunt by filling in with the Klenow fragment of DNA polymerase I prior to the second ligation. Transformed *E. coli* JM109 colonies were screened by PCR to identify the presence of the appropriate insert. The constructed recombinant plasmids were verified by dideoxy chain termination DNA sequencing using a T7 DNA polymerase-based sequencing kit (Pharmacia Biotech Inc.). The construction of the expression plasmids is summarized in Figure 1.

Rapid Large-Scale Purification of the Chicken TnT1 NH_2-Terminal Fragments

Purification of N47. Four liters of 2× tryptone-yeast broth containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol was inoculated with a single colony of *E. coli* BL21-(DE3)pLysS (Studier *et al*., 1990) transformed with pTx47. The culture was incubated at 37 °C with vigorous shaking until $OD_{600} = 0.4-0.5$, induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and incubated an additional 3 h. The bacteria were harvested by centrifugation, resuspended in 50 mL of 0.1 M sodium phosphate buffer, pH 7.0, and lysed by three passes through a French press at 1000 psi. The lysate was heated to 80 °C, immediately chilled on ice for 1 h, and centrifugated to pellet the insoluble proteins. To selectively separate the metal-binding N47 TnT fragment from the bacterial lysate proteins, the supernatant was loaded onto a 10 mL column of Chelating Fast Flow Sepharose (Pharmacia LKB Technology) charged with Zn^{2+} and equilibrated in 0.5 M NaCl, 0.1 M sodium phosphate buffer, pH 7.0 (Jin & Smillie, 1994). The column was washed with 2 bed volumes each of 5 and 10 mM imidazole and eluted by a stepwise gradient, with the N47 protein eluting at imidazole concentrations greater than 40 mM, as analyzed by SDS−polyacrylamide gel electrophoresis (PAGE). The N47 peak was further fractionated by a Sephadex G75 column (2.5 cm × 125 cm) equilibrated with 20 mM sodium

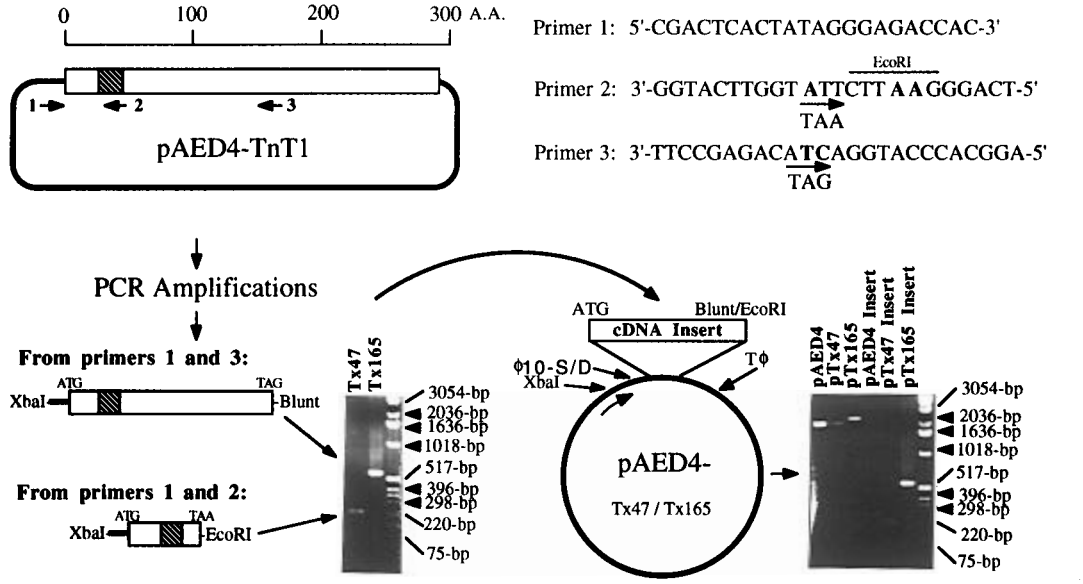

FIGURE 1: Cloning of chicken TnT1 NH_2-terminal cDNA fragments into the expression vector. Using three unique synthetic oligonucleotide primers, NH_2-terminal cDNA fragments encoding 47 (N47, primers 1 and 2) and 165 (N165, primers 1 and 3) amino acids were amplified by PCR from an expression plasmid encoding chicken TnT isoform 1 (shown in the left insert of 0.9% agarose gel). Primers 2 and 3 were designed to introduce translation termination codons through minimal base pair mismatches (seen in bold letters) while primer 2 also encodes a 3′ *Eco*RI restriction endonuclease site. Both cDNA fragments were inserted unidirectionally and in-frame into the pAED4 expression plasmid following agarose gel purification. The constructed expression plasmids were analyzed by agarose gel electrophoresis sizing as well as PCR to confirm the presence of the respective cDNA inserts (shown in right insert of 0.9% agarose gel). ϕ10-S/D, coupled T7 and Shine-Dalgarno ribosomal binding sequence; Tϕ, transcription terminator.

phosphate buffer, pH 7.0. Gel filtration fractions were examined by SDS−PAGE and those containing pure N47 were lyophilized after dialysis against 0.5% formic acid for three changes. The high level expression of N47 (Figure 2), although a low molecular weight peptide, yielded 10 mg (2 μmol) of highly purified protein per liter of bacterial culture, providing sufficient material for characterization.

Purification of N165. Four liters of pTx165 transformed *E. coli* BL21(DE3)pLysS culture were set as above and induced by 0.4 mM IPTG at $OD_{600} = 0.6-0.7$ and further incubated for only 2.5 h. Adopted from an *E. coli* acetone powder method (L. B. Smillie, unpublished results), harvested bacteria were extracted three times with cold ethanol and acetone. The *E. coli* acetone powder was briefly dried and solubilized in 100 mL of 6 M urea, 50 mM Tris-HCl, pH 9.2, with stirring at 4 °C. After pH adjustment to 7.4 and centrifugation, the supernatant was loaded onto a 10 mL Zn^{2+}-affinity column equilibrated as above with the addition of 6 M urea. Following washing, the N165 protein was eluted only at imidazole concentrations greater than 40 mM, as analyzed by SDS−PAGE. The peak fractions were dialyzed against 50 mM ammonium bicarbonate and lyophilized. The dried powder was dissolved in 0.5 M NaCl, 20 mM sodium phosphate buffer, pH 7.0, 0.05% NaN_3 and loaded onto a 1.6 cm × 50 cm Superose 12 column monitored by a Pharmacia Biotech Inc. FPLC system controlling flow rate at 0.3 mL/min. The A_{280nm} peaks of the fractions were examined by SDS−PAGE and those containing pure N165 were collected (Figure 3), dialyzed against 50 mM ammonium bicarbonate, and lyophilized.

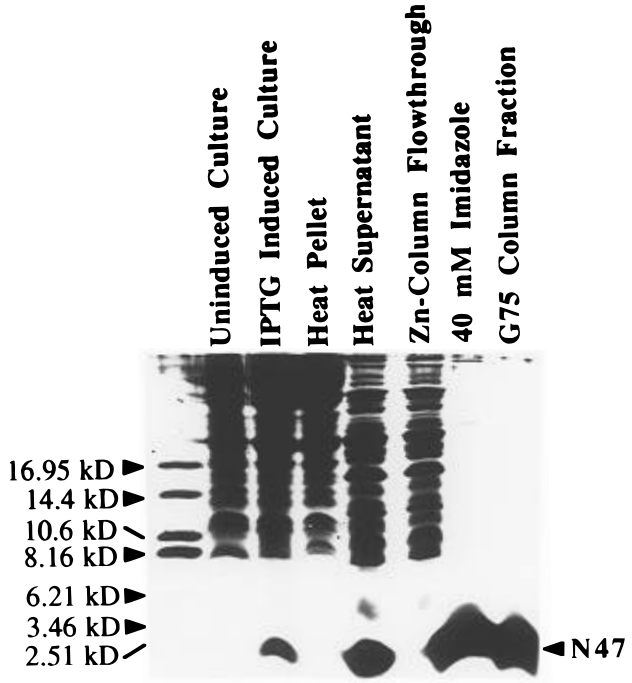

FIGURE 2: Expression and purification of TnT fragment N47. SDS−PAGE using 14% small-pore gels and Tris-Tricine running buffer was performed to monitor the expression and purification of N47, as described previously (Jin, 1995). The N47 protein band was visible in the lysate of the bacterial culture following induction with IPTG. The French press lysate was heated to 80 °C, cooled on ice, and centrifuged to remove the majority of the contaminant bacterial proteins precipitated by heat denaturation. N47 in the soluble fraction was effectively recovered by Zn^{2+}-affinity chromatography. G-75 gel filtration chromatography further purified N47 to homogeneity.

Authenticity of the Genetically Expressed TnT Fragments

Amino Acid Analysis, Peptide Sequencing, and Mass Spectrometry. Amino acid analysis of N47 as well as five cycles of NH_2-terminal amino acid sequencing were done by the Protein Sequencing Facility, University of Calgary Faculty of Medicine. Amino acid analyses of N165 and various CD samples were generously carried out by Dr. J.

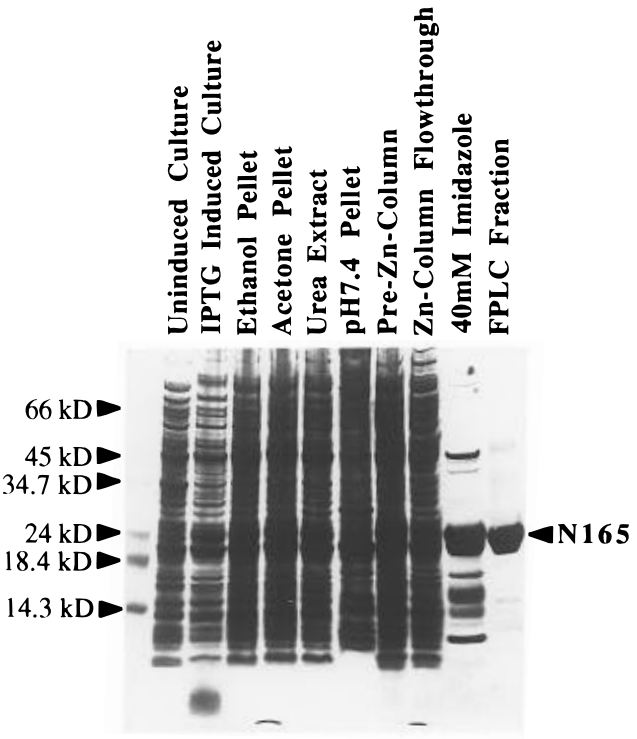

FIGURE 3: Expression and purification of TnT fragment N165. SDS−PAGE using 12% Laemmli gels was performed as described previously (Jin, 1995) to examine the expression and purification of N165. Following induction of the culture with IPTG, bacterial cells were pelleted and washed with ethanol and acetone to provide a lipid-free protein powder. Following extraction with 6 M urea, 50 mM Tris-HCl, pH 9.2, the soluble fraction was adjusted to pH 7.4, and loaded on a Zn^{2+}-affinity column. Specifically retained N165 was eluted from the column by imidazole and further fractionated from other contaminant bacterial proteins by a Superose 12 FPLC column.

R. Pearlstone and M. R. Carpenter in the laboratory of Dr. L. B. Smillie, University of Alberta. Mass spectrometry analysis of the sample of chicken TnT1 fragment N47 was performed by the Alberta Peptide Institute, University of Alberta, Edmonton, Canada. The experimentally determined amino acid compositions of N47 and N165 were in good agreement with the ratios predicted from the nucleotide sequences. Mass spectrometry of N47 displayed a single sharp peak at molecular weight 5254.10, in correspondence with the calculated molecular weight of N47, minus the initial methionine residue at position 1 (M_r = 5253). This observation agrees with the amino acid analysis of N47, in which no methionine residue was recovered. NH_2-terminal amino acid sequencing of the purified N47 protein indicated that Ser, rather than Met, was the first amino acid released, followed by Asp-Thr-Glu-Glu, confirming the expression of the correct peptide and the high efficiency removal of the initial methionine of this chicken TnT fragment expressed in *E. coli* BL21.

Western Blotting. Using the cloned, bacterially expressed Tx-positive chicken breast muscle TnT isoform as a control (TnT1; Smillie *et al.*, 1988; Jin *et al.*, 1991), both N165 and TnT1 were resolved by 12% SDS−PAGE and transferred to nitrocellulose membrane (0.22 μm) using a Bio-Rad semidry electrotransfer apparatus. After overnight blocking with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.5), the nitrocellulose membranes were incubated at 4 °C overnight in TBS containing 0.1% BSA with either rabbit polyclonal antisera raised against chicken TnT (RATnT) or the Tx 20mer peptide (RATx) (Jin & Smillie, 1994). The following washes, incubation with alkaline phosphatase-labeled goat anti-rabbit IgG second antibody (Sigma), as well as development of the 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP−NBT) color reaction were performed as described (Ausubel *et al.*, 1995). The results demonstrate that both N165 and TnT1 were identified by the two antisera (figure not shown), confirming the TnT origin of N165 as well as the presence of the Tx segment.

Indirect Enzyme-Linked Immunosorbant Assay. The authenticity of N47 was established by indirect enzyme-linked immunosorbant assay (ELISA) performed with the TnT-specific RATnT and Tx element-specific RATx antisera. In these experiments, native Tx-positive TnT was prepared from chicken breast muscle by conventional methods (Pearlstone *et al.*, 1977) for use as a control. Modified from a method described previously (Jin & Lin, 1988), wells of microtiter plates (Falcon 3915) were coated overnight at 4 °C with chicken breast muscle TnT, TnT fragment N47, or BSA dissolved to 5 μg/mL in 50 mM carbonate buffer, pH 9.6. The plates were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4) plus 0.05% Tween-20 (PBS-T) and blocked for 2 h at room temperature with PBS-T plus 1% BSA. After being washed with PBS-T, the plates were incubated for 3 h at 37 °C with serial dilutions of RATnT or RATx in PBS-T plus 0.1% BSA, washed with PBS-T and incubated with 1:1,500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG second antibody (Sigma) in PBS-T plus 0.1% BSA for 40 min at 37 °C. The plates were washed with PBS-T and H_2O_2-2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate in 0.1 M citrate buffer, pH 4.0, was added for colour development at room temperature. The microtiter plates were monitored for absorbance at 405 nm by a Bio-Rad model 3550UV automated microplate reader. The results demonstrate positive titration curves for both RATnT and RATx antibodies against the cloned N47 peptide (figure not shown).

Analytical Zn^{2+}-Affinity Chromatography of TnT1 and Its Fragments. To compare the relative Zn^{2+}-binding affinity of the engineered TnT proteins, aliquots of cloned TnT1, TnT3, N165, N47, and the Tx 20mer peptide were dissolved in 0.5 M KCl, 0.1 M sodium phosphate buffer, pH 7.0, and loaded onto a 2 mL Zn^{2+}-affinity Chelating Fast Flow Sepharose column (Jin & Smillie, 1994) equilibrated in the same buffer. After being washed with 3 mL of the equilibration buffer, the column was eluted by a linear gradient of 2 to 80 mM imidazole (78 mL total volume) in the above buffer. The specific proteins eluted were identified by Laemmli and small-pore SDS−PAGE analyses (Jin, 1995) of the fractions. The Coomassie Blue R250-stained gels were scanned by a Pharmacia Image Master Desktop densitometer and the calculated (OD × area) values of the specific protein bands were used to judge the peak position. A summary of the TnT functional domains and the NH_2-terminal variable region organization of chicken breast muscle TnT, TnT1, and TnT3 isoforms, as well as the sequence of the Tx 20mer peptide, are illustrated in Figure 4.

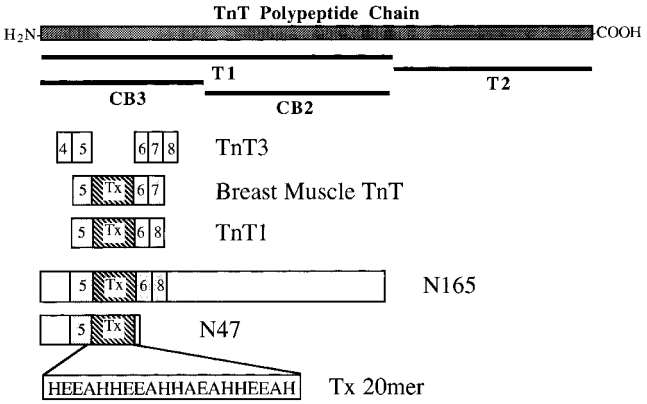

FIGURE 4: Functional domains of TnT, the NH_2-terminal variable region of chicken breast muscle TnT, TnT1, TnT3, and the sequence of the Tx peptide. Regions of the T1, T2, CB2, and CB3 fragments (Pearlstone & Smillie, 1982) of TnT are outlined corresponding to the whole polypeptide chain. The NH_2-terminal exon organization of the chicken TnT3, breast muscle TnT and TnT1, structures of N165, N47, and the Tx peptide derived from TnT1 are also shown in this figure.

Circular Dichroism Analysis of N47 and N165

Aliquots of the purified chicken TnT1 NH_2-terminal fragments N47 and N165 were equilibrium-dialyzed separately at 4 °C overnight for two changes against 500 volumes of 0.1 M KCl, 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, $ZnCl_2$, $CoCl_2$, $NiCl_2$, or $CuCl_2$. Circular dichroism (CD) measurements were carried out at 25 °C on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) calibrated with ammonium *d*-(+)-10-camphorsulfonate at 290.5 and 192 nm, and with *d*-(−)-pantoyllactone at 219 nm. Using a 0.02 cm cell, each sample was scanned 10 times, in the presence and absence of 2,2,2-trifluoroethanol (TFE) to 50% (v/v). Noise reduction was applied to remove the high frequency before calculating molar ellipticities. Protein concentrations were quantitated by amino acid analysis upon completion of CD measurements. Calculation of the secondary structure contents of the tested samples was as described (Tusnady *et al*., 1991).

Epitopic Structure Characterization of TnT Isoforms and Fragments

Monoclonal Antibody Epitope Analysis. Previous experiments characterizing a monoclonal antibody (mAb) against chicken *h1*-calponin, CP3, showed its specific cross-reaction to cloned TnT1 and chicken breast muscle TnT, both Tx-positive chicken TnT isoforms. Interestingly, this mAb did not show cross-reaction to the Tx-negative TnT2 (Smillie *et al.*, 1988) or the TnT isoforms found in chicken leg muscle (Jin *et al.*, 1996). To further elaborate and define this unique cross-reaction to the Tx-positive TnTs, indirect ELISA was performed as described earlier using mAb CP3 on N47, N165, TnT3, chicken breast muscle TnT, as well as a limited chymotryptic digest of chicken breast muscle TnT to yield the T1 and T2 fragments (Figure 4; Pearlstone & Smillie, 1985).

Effect of Zn^{2+} on the Epitopic Structure of the Tx Segment. To determine the conformational changes as a consequence of Zn^{2+}-binding to the Tx element in the NH_2-terminal region of TnT1, indirect ELISA was done using TnT1 pretreated with varying concentrations of $ZnCl_2$. TnT1 was dissolved (5 μg/mL) in a modified actin binding buffer (Buffer A; 0.1 M KCl, 10 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$) and coated overnight at 4 °C onto triplicate wells of microtiter plates (Falcon 3915; 100 μL/well) containing 0.02, 0.04, 0.06, 0.08, 0.1 mM $ZnCl_2$, or 0.1 mM EDTA. After blotting the wells dry, the plates were blocked at room temperature for 2 h with Buffer A plus 1% BSA and 0.05% Tween-20. The wells were washed three times with Buffer A plus 0.05% Tween-20 (Buffer T) and serial dilutions of the Tx segment-specific antibody (RATx) in Buffer T plus 0.1% BSA were added to the plates (100 μL/well) and incubated for 1 h at room temperature. Following washing with Buffer T, the plates were incubated with 1:750 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG second antibody (Sigma) for 40 min at room temperature. For color development, the plates were washed with Buffer T and incubated with H_2O_2-ABTS substrate as outlined before.

Solid-Phase Tropomyosin-Binding Assay for TnT Isoforms and Fragments

The binding of N47, N165, the Tx-positive chicken breast muscle TnT and Tx-negative TnT3 isoform to α-Tm was compared by an ELISA-mediated protein binding assay (Jin, 1995). In performing the Tm-binding assays, TnT isoforms and fragments were coated onto microtiter plates in Buffer A containing 0.1 mM $ZnCl_2$ or 0.1 mM EDTA; BSA coated under identical conditions was used as a nonspecific protein binding control. The plates were blocked and washed as before, and incubated for 2 h at room temperature with Buffer A containing serial dilutions of rabbit α-Tm, prepared as described (Heeley *et al.*, 1989). After washing three times with Buffer T, an anti-Tm specific mAb CH1 (generously provided by Dr. Jim Lin, University of Iowa) diluted 1:1000 in Buffer T plus 0.1% BSA was added to the plates and incubated at room temperature for 1 h. The Tm associated with the TnT coated on the plate was detected through the bound anti-Tm mAb. The following washes, horseradish peroxidase-conjugated anti-mouse IgG secondary antibody incubation and H_2O_2-ABTS substrate color development were performed as described earlier.

RESULTS

Effects of Metal-Binding on the Secondary Structure of the NH_2-Terminal Fragments of TnT1

CD spectroscopy of N47 (Figure 5) under non-denaturing conditions indicated this TnT NH_2-terminal fragment to be predominantly random folded with a low percentage of α-helical content (4.2%−10.5%) when in the presence of EDTA, Zn^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+}. The addition of TFE to 50% to N47 (N47-TFE) enhanced α-helical secondary structure under the metal-free conditions (24.3%). N47-TFE samples in the presence of Zn^{2+} showed α-helical content of 21.4%, similar to that in the presence of EDTA, whereas samples with Cu^{2+}, Ni^{2+} or Co^{2+} displayed significantly higher α-helical content, ranging from 49.8% to 59.8%.

The CD spectroscopy results of the N165 fragment of chicken TnT1 are shown in Figure 6. Under non-denaturing conditions, N165 showed α-helical content up to 30.9%, comparable to previous data for the T1 fragment of rabbit fast skeletal muscle TnT (Pearlstone & Smillie, 1985). The equilibrium dialysis against Zn^{2+}, Co^{2+} or Ni^{2+} did not produce significant effects on the overall secondary structure

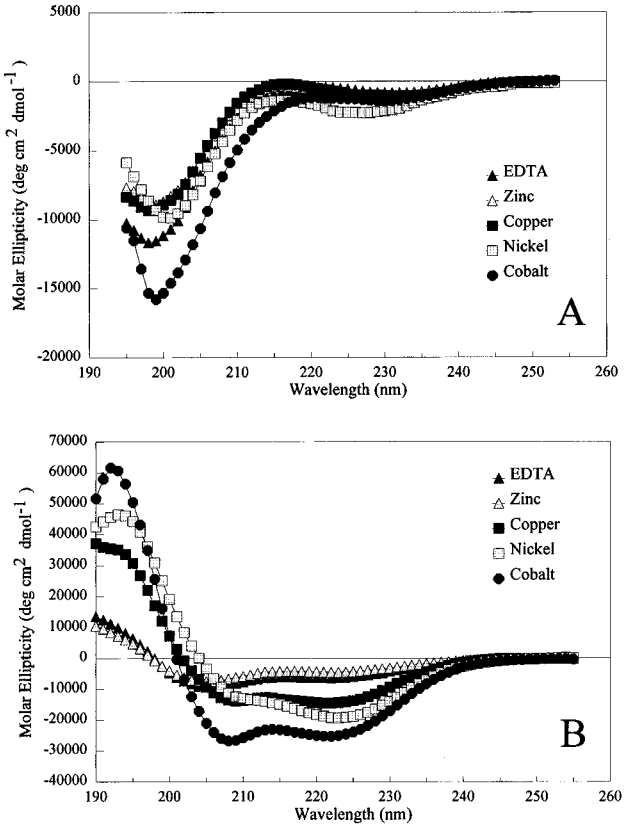

FIGURE 5: Circular dichroism analysis of N47. Circular dichroism measurements of N47 in 0.1 M KCl, 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, Zn^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+} (A) showed predominantly random folding. Addition of TFE (B) to 50% (v/v) demonstrated an increase in α-helical character of all samples tested, notably significant in the presence of Co^{2+}, Ni^{2+}, and Cu^{2+} (Table 1, part A).

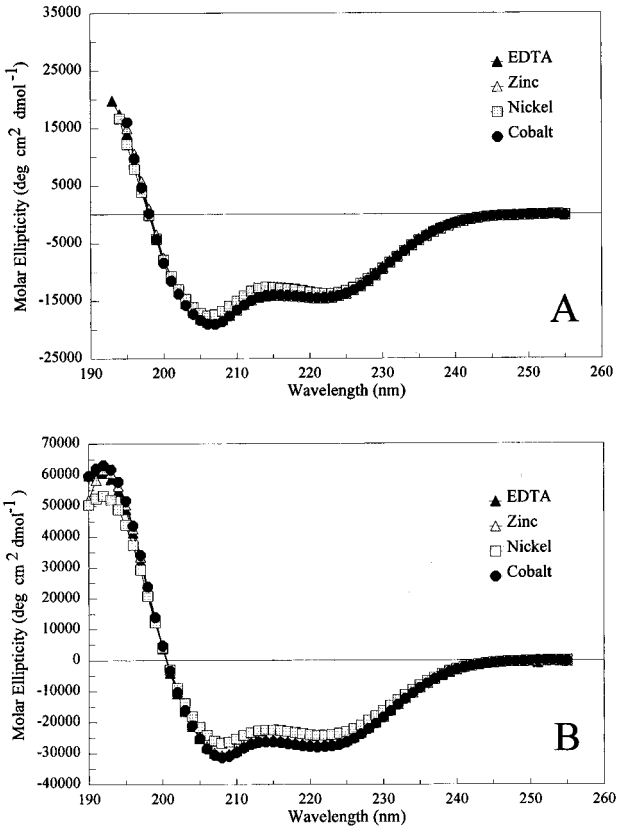

FIGURE 6: Circular dichroism analysis of N165. N165 in 0.1 M KCl, 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, Zn^{2+}, Co^{2+}, or Ni^{2+} (A) had similar α-helical content to previously published data on the T1 fragment of rabbit fast skeletal muscle TnT. With the addition of TFE (B) to 50% (v/v), α-helical content was further stabilized without significant differences seen in the absence or presence of different metals (Table 1, part B).

contents of N165 in the benign buffer. The addition of TFE to 50% (N165-TFE) increased the α-helical character of all samples tested (52.2%−70.1%). In contrast to the significant differences observed between the spectra of N47 in the presence of TFE and specified transition metals, N165 in the presence of various metals showed insignificant changes of the overall α-helical content as compared to that in EDTA. The secondary structure contents of N47 and N165 under different conditions are summarized in Table 1, sections A and B, respectively.

Effect of Zn^{2+} on the Epitopic Structure of the Tx Segment in the NH_2-Terminal Region of TnT1

As the transition metal of most biological importance, Zn^{2+} was further studied for its effect on the structure and function of Tx-positive TnT. Figure 7 shows that binding with Zn^{2+} had significant effects on the epitopic structure of the Tx cluster in the NH_2-terminal region of TnT. The reactivity of the Tx segment-specific RATx antibody to TnT1 decreased as the concentration of Zn^{2+} during pretreatment was increased, as compared to when 0.1 mM EDTA was present to deplete divalent ions. This effect was not confined to the genetically expressed TnT1 isoform, as chicken breast muscle TnT coated under identical conditions showed similar results (data not shown). This change in anti-Tx antibody reactivity demonstrates that the binding of Zn^{2+} modifies the three-dimensional structure of the Tx segment within the intact TnT polypeptide. The concentration of Zn^{2+} required to produce 50% of the maximum inhibitory effect was determined from this titration to be 41 μM. In separate ELISA experiments, we have observed that the low concentration (0.1 mM) of Zn^{2+} or EDTA had no nonspecific effects on the coating of a protein to the microtiter plates or immunoidentification of the protein (data not shown).

Metal-Binding Related Conformation of the Tx-Containing TnT and its NH_2-Terminal Fragments

Zn^{2+}-Binding Avidity. The chromatographic binding comparison of TnT1, N165, N47, and Tx 20mer demonstrated that the intact TnT1 isoform had a difference in binding to the column as compared to its fragments (as indicated by the imidazole concentration required for their elution). The TnT1 NH_2-terminal fragments ranging from 20 amino acids to a half protein (165 amino acids) all eluted near 60 mM imidazole. In contrast, intact TnT1 eluted at significantly lower imidazole concentrations (30−36 mM), indicating an interaction between the COOH-terminal domain and the Tx element in the NH_2-terminal variable region. As a negative control, TnT3, which lacks the Tx segment in the NH_2-terminal variable region (Figure 4; Smillie *et al.*, 1988), showed no specific binding and appeared in the flowthrough fraction as expected. These results, along with physical data of the proteins/peptides, are summarized in Table 2.

mAb Epitopic Structures. As previously characterized, the anti-*h1*-calponin mAb CP3 specifically recognizes the Tx-positive TnT1 and chicken breast muscle TnT without cross-

Table 1: Secondary Structure Contents of the Chicken TnT1 NH_2-Terminal Fragments

	α helix		β sheet		β turn		unordered		other	
buffer	−TFE	+TFE	−TFE	+TFE	−TFE	+TFE	−TFE	+TFE	−TFE	+TFE
					(A) N47					
EDTA	4.2	24.3	0.5	0.3	39.9	33.7	52.9	37.9	2.5	3.8
zinc	8.0	21.4	0.4	0.5	45.5	37.2	46.0	37.3	0.1	3.5
cobalt	4.2	59.8	11.1	15.4	21.0	0.0	63.7	24.8	0.1	0.0
nickel	9.7	53.6	0.0	13.4	28.0	32.7	61.5	0.0	0.0	0.1
copper	6.3	49.8	0.2	8.9	46.8	10.2	46.6	27.7	0.2	3.5
					(B) N165					
EDTA	29.6	66.2	13.0	5.9	3.2	0.0	53.5	27.9	0.8	0.0
zinc	30.9	67.9	13.2	6.4	1.5	0.0	54.0	25.7	0.5	0.0
cobalt	30.8	70.1	11.8	4.2	1.4	0.0	56.1	25.7	0.0	0.0
nickel	23.9	52.2	35.6	17.2	10.1	0.0	30.4	30.4	0.0	0.1

[a] Secondary structure contents were calculated from the CD spectra, according to the method described by Tusnady *et al.* (1991). Values are presented as a percentage, with (+) or without (−) the addition ot TFE to 50% (v/v).

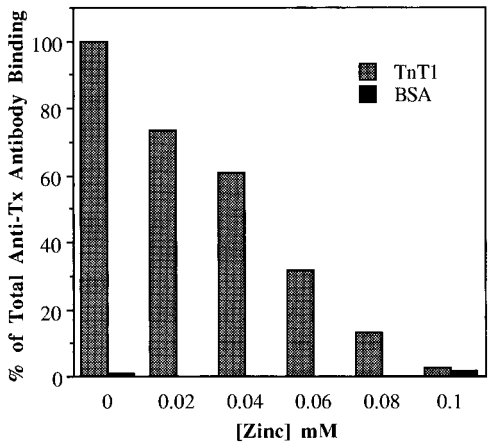

FIGURE 7: Effect of Zn^{2+} ions on the epitopic structure of TnT1 recognized by RATx. TnT1 was coated on ELISA plates in the presence of 0.02, 0.04, 0.06, 0.08, 0.1 mM $ZnCl_2$ or 0.1 mM EDTA. After blocking, the wells were incubated with serial dilutions of the RATx antiserum which is specific against the Tx peptide. Following incubation with horseradish peroxidase-labeled goat anti-rabbit IgG second antibody, H_2O_2-ABTS substrate was added for color development and monitored for absorbance at 405 nm. The values are plotted as a percentage of total antibody binding, with the 0.1 mM EDTA sample assumed to be 100% binding without Zn^{2+} effects. BSA coated onto the plates is used as a negative control. The results show a linear effect of increased Zn^{2+} concentration on the decrease of RATx epitope recognition.

Table 2: Physical Data and Zinc-Binding Affinity of the TnT Isoforms and Fragments

protein	amino acid residues	molecular weight	isoelectric point	[imidazole] at peak elution
TnT3	1−263 (−Tx)	31 342	6.49	(flowthrough)
TnT1	1−274	32 340	6.64	30−36 mM
N165	1−165	19 521	4.99	54−60 mM
N47	2−47	5384	4.48	57−63 mM
Tx 20mer	19−38	2416	5.36	60−66 mM

[a] Molecular weights and isoelectric points were calculated theoretically from the amino acid sequences. The imidazole concentrations required for elution were assigned from the analytical zinc-affinity chromatography assay.

reaction to chicken TnT2 or any other Tx-negative TnT isoforms in the leg muscle (Jin *et al.*, 1996). To demonstrate the nature of the cross-reaction of CP3 to the Tx-positive TnTs, indirect ELISA was done on N47, N165, TnT3, intact chicken breast muscle TnT and a mixture of the T1 and T2 fragments of chicken breast muscle TnT prepared by limited chymotrypsin digestion. As shown in Figure 8, neither N47

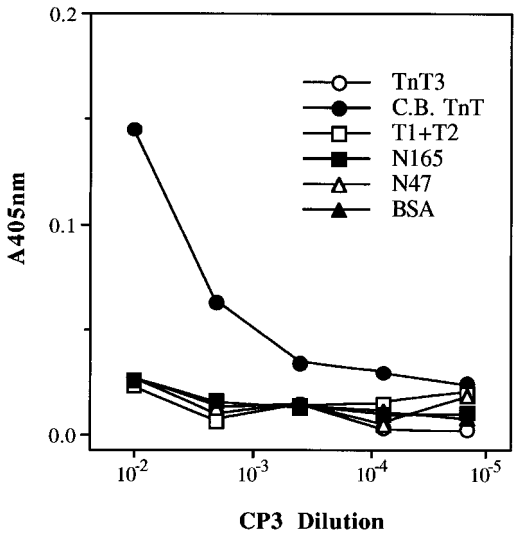

FIGURE 8: mAb CP3 epitope recognition of Tx-positive TnT and its fragments. Indirect ELISA experiments were performed, as described in Figure 4, on the Tx-positive chicken breast (C.B.) muscle TnT, a mixture of its chymotryptic T1 and T2 fragments, as well as N47, N165 and TnT3 using serial dilutions of mAb CP3. The results indicate that only the intact breast muscle TnT is recognized by the mAb, indicating that the epitope structure is dependent on both the presence of the Tx segment and an intact TnT polypeptide chain.

or N165 were reactive to the mAb, indicating that the CP3 epitope is not the Tx structure itself. Intact chicken breast muscle TnT was recognized but the mixture of its T1 and T2 fragments was not reactive. TnT3, a Tx-negative isoform identical to TnT1 except in the NH_2-terminal region, is not recognized by CP3. These results demonstrate that the CP3 epitope is not simply the T1/T2 junction, a conserved region in all chicken fast TnTs. The data, therefore, suggest that the CP3 epitope structure locates outside the Tx segment but is induced by the NH_2-terminal Tx structure, an effect that requires an intact TnT polypeptide chain.

Effect of Zn^{2+} on the Interaction of Tx-Positive TnT or Fragments and Tm

The pretreatment of chicken breast muscle TnT with Zn^{2+} produced a significant difference in its binding to Tm. Saturation of the Zn^{2+}-binding sites on the Tx element of the intact TnT dramatically decreased the TnT−Tm interaction, as demonstrated by the ELISA-mediated protein-binding assay (Figure 9A). In contrast, Zn^{2+} treatment had no

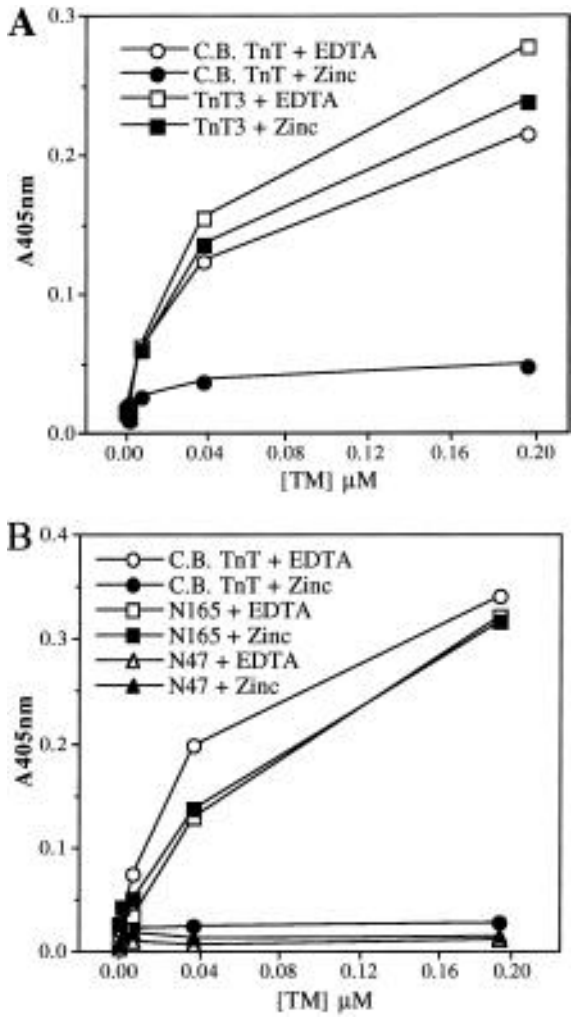

FIGURE 9: Effect of Zn^{2+} on the association of chicken breast muscle TnT with α-Tm. (A) Chicken breast (C.B.) muscle TnT (Tx-positive) or TnT3 (Tx-negative) were coated in the presence of 0.1 mM $ZnCl_2$ or 0.1 mM EDTA. After blocking, the plates were incubated with serial dilutions of rabbit α-Tm, followed by anti-Tm mAb CH1 to detect the bound Tm. The following washing, secondary antibody incubation and color development steps were performed as described in Figure 7 to reveal the TnT−Tm interaction. Simultaneous experiments with BSA coated on the plates were used as the control for nonspecific protein bindings. (B) Effects of Zn^{2+} pretreatment on the interaction of N47, N165, and chicken breast muscle TnT with α-Tm were further demonstrated in separate experiments. For all experiments, Tm concentrations were calculated based on the molecular weight of the monomer protein. The results demonstrate a Zn^{2+}−Tx-binding-induced decrease in the interaction of intact TnT with Tm, evidently requiring the presence of the COOH-terminal domain that is absent from the N165 fragment.

significant effect on the Tx-negative TnT3−Tm interaction as compared to the EDTA control. N47 showed no binding to Tm under either condition tested, in agreement with previous data characterizing the comparable CB3 fragment (Pearlstone & Smillie, 1982). Tm bound to N165 (equivalent to the T1 fragment of TnT, Figure 4) pretreated with EDTA, but the interaction was not as strong as that seen with the intact TnT isoforms, evidenced by the sharper decrease in its association when incubated with lower concentrations of Tm (Figure 9B; compared with binding to chicken breast muscle TnT, ∼2-fold higher concentration of Tm was required for 50% of the maximum binding to N165). This decrease in N165's interaction with Tm reflects the contribution of the high affinity COOH-terminal Tm-binding site (Pearlstone & Smillie, 1982) of the intact TnTs. Pretreatment of N165 with Zn^{2+} did not produce significant changes in its binding to Tm through the central domain binding site. Therefore, the significantly decreased binding of Tm to the chicken breast muscle TnT after Zn^{2+} pretreatment ($P < 0.01$) involves changes to both the central and COOH-terminal domains of TnT, that is induced by changes of the Tx element's conformation (Figure 7) in the NH_2-terminus, which may in turn affect the overall structure of intact TnT (Figure 8). The insignificant variation of the TnT3 association with Tm in the presence and absence of Zn^{2+} ($P > 0.05$) might be due to interactions between Zn^{2+} and lone amino acids (i.e. His, Cys) or pockets of negative charge on the proteins, which may affect the conformation and activity of TnT.

DISCUSSION

Metal-Induced Secondary Structure Changes in the NH_2-Terminal Region of Chicken TnT1. The CD measurements shed light on an interesting feature of the Tx element. TnT fragment N47, in the presence of TFE and EDTA or Zn^{2+}, is calculated to be 21.4 - 24.3% α-helical (Table 1, part A). In the presence of Co^{2+}, Ni^{2+}, or Cu^{2+}, N47-TFE shows a 2-fold increase in α-helical content. This is a novel demonstration that metal-binding may stabilize α-helical structure. The results suggest that the metal-inducible α-helical character of the Tx element is dependent upon the intrinsic characteristics of the metal ion rather than the ion's affinity for the Tx element, as Zn^{2+} and Co^{2+}, which show similar affinities (Jin & Smillie, 1994), demonstrate 2-fold differences in α-helical content in the presence of TFE. Although the mechanism of effect of TFE has not been conclusively established, several possible models are proposed (Llinas & Klein, 1975; Nelson & Kallenbach, 1986). Using synthetic actin NH_2-terminal peptides, Sönnichsen *et al.* (1992) have demonstrated that TFE acts primarily as a helix-enhancing cosolvent as opposed to a helix-inducing solvent. Other studies using protein fragments and peptides corresponding to α-helical regions in proteins showed strong correlation between the TFE-enhanced structure and the native protein structure (Leist & Thomas, 1984). The enhanced α-helical content seen for N47 in the presence of TFE and Co^{2+}, Ni^{2+} or Cu^{2+} is not observed in the presence of TFE and EDTA or Zn^{2+} (Table 1, part A), arguing directly against the action of TFE as the lone cause for the phenomenon. It has been shown, however, that changes in α-helical character initiated by the binding of metal ions may indicate a potential rearrangement of helices (Dedman *et al.*, 1977; Babu *et al.*, 1985; Herzberg *et al.*, 1986). Although the resolution provided by the CD measurements is not sufficient to conclude the nature of the conformational change, an effect of transition metals on the overall structure of this TnT fragment is demonstrated.

Under non-denaturing conditions, the α-helical content of N165 is in the range of 30%, comparable to data on the chymotryptic fragment T1 of rabbit fast skeletal muscle TnT (Pearlstone & Smillie, 1985), indicating that N165 is a stable half-molecule. TFE enhanced α-helical character in all samples of N165 tested, whereas the presence of metal ion did not indicate significant overall changes in α-helical content. Nonetheless, a local secondary structure change induced by metal ions in the region containing the Tx segment (like that shown by the N47 fragment) may not be

adequate to generate a detectable variation in the α-helical content or overall structure of N165.

Metal-Induced Tertiary Structure Changes in the Tx Segment. As shown in Figure 7, Zn^{2+} is able to induce conformational changes of the NH_2-terminal Tx segment in the TnT1 molecule, as detected by the Tx segment-specific antibody. The RATx polyclonal antiserum presumably recognizes multiple epitopes formed by the Tx segment and the significantly reduced antibody binding avidity due to the binding of Zn^{2+} suggests an extensive tertiary structure change in the Tx segment locating in the NH_2-terminal region of the intact TnT protein. For proteins such as TnT whose crystal or NMR three-dimensional structure is not available, the antibody epitope assay provides a useful method to quantitatively monitor conformational changes under various conditions (Goldberg, 1991).

Interactions between the NH_2-Terminal Variable Region and Other Domains of Intact TnT. A very interesting phenomenon is demonstrated by the Zn^{2+}-binding avidity comparison (summarized in Table 2), as well as the epitopic recognition exhibited by the CP3 mAb. The Zn^{2+}-binding avidity difference between intact TnT1 and its Tx-containing fragments indicates possible tertiary interactions between the NH_2-terminal region and the COOH-terminal domain of TnT, which may affect the mode of Tx's metal-binding in intact TnT1. The mAb CP3 has shown specific cross-reactivity to TnT1 and chicken breast muscle TnT (both Tx-positive) without cross-reactivity to TnT2 and the leg muscle TnT isoforms (Jin *et al.*, 1996), which are all Tx-negative but have identical primary structure in the COOH-terminal and central domains as the breast muscle TnTs, including identical primary structure in the T1/T2 junction. Our results indicate that the epitope is eliminated when the intact TnT is cleaved into T1/T2 fragments. Since TnT3 has a T1/T2 junction identical in sequence, but remains unrecognized, these results clearly demonstrate that the epitope recognized by CP3 is not the Tx segment itself but due to the overall three-dimensional conformation of intact Tx-positive TnT, which does not appear to be a simple addition of the NH_2- and COOH-terminal domains, but depends on an intact TnT polypeptide chain.

Effect of Zn^{2+}-Induced Structural Changes on TnT's Tm-Binding Function. In addition to inducing changes in the NH_2-terminal and overall conformation of the metal-binding TnT, the effect of Zn^{2+} on the association of TnT with Tm is shown in Figure 9A. While chicken breast muscle TnT and Tm specifically interact in a metal depleted environment, Zn^{2+}-binding to the Tx element demonstrates significant consequences for this association, potentially through tertiary interactions between the NH_2- and COOH-terminal domains. The insignificant effect of Zn^{2+} pretreatment on N165's association with Tm indicates the central domain Tm-binding site is not affected when the COOH-terminal domain is absent. The difference in tropomyosin-binding of N165 and intact TnT (Figure 9B) in response to the metal-induced NH_2-terminal conformational change agrees with the established two-site TnT−Tm interaction model (Pearlstone & Smillie, 1982). The dramatic inhibition of the interaction between Zn^{2+}-pretreated chicken breast muscle TnT and Tm demonstrates induced structural changes in both the central and COOH-terminal structure of TnT propogated by Zn^{2+}-binding to the NH_2-terminal Tx segment. These structural changes in regions with defined roles in the TnT−Tm interaction depend on the intact TnT polypeptide chain. As a physiological consequence, Zn^{2+} in the chicken breast myofibril environment would modify the association of TnT with Tm, and may in turn affect the thin filament-linked Ca^{2+}-regulation of contraction. The significant difference in total Zn^{2+} content between chicken breast (pectoralis major; 0.26 mmol/kg dry weight) and gastrocnemius muscle (0.52 mmol/kg dry weight; a value similar to that observed in several other Tx-negative avian and mammalian muscles tested) has been previously demonstrated (Jin & Smillie, 1994). The dramatic inhibition of TnT−Tm interaction resulting from Zn^{2+} binding to the Tx element indicates that Zn^{2+} accumulation in the Tx-positive TnT-containing myofibrils may in fact be detrimental to the mechanism of muscle contraction. Therefore, the significantly lowered Zn^{2+} contents of the chicken breast muscle when compared to the Tx-negative muscles ($P < 0.01$) may reflect a protective adaptation mechanism to limit the concentration of Zn^{2+} in the sarcomeric environment.

Data from this present study further suggest that the structural changes initiated by the variable NH_2-terminal region may influence the structure and function of other domains of TnT, particularly the regions which bear established Tm-binding activity. TnT is believed to have a highly extended shape and the mechanism for the interaction between the NH_2- and COOH-terminal domains within the intact polypeptide chain needs to be further investigated. Through subtle changes in the overall conformation of TnT molecule, this mechanism may execute the functional significance of TnT isoforms with primary structure differences in the NH_2-terminal region and may also contribute to the phenotype of TnT point mutations, causing the chronic development of cardiomyopathies (Watkins *et al.*, 1995). With potential interactions within the intact TnT molecule, the contribution and functional significance of the alternatively spliced NH_2-terminal variable region may not be potentiated in studies using TnT fragments. Therefore, experiments characterizing intact TnT isoforms would be more physiologically meaningful and may be expected to show subtle differences insofar as their role and action in the regulation of the striated muscle thin filament. As a prime example, the binding of metal ions to the variable NH_2-terminal region of the Tx-positive TnT isoforms is able to induce a specific reconfiguration of the TnT molecule and further affect the function of TnT, providing a novel approach to study the role of TnT isoforms in the regulation of muscle contraction.

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