Weeden, N. F. (1981) J. Mol. Evol. 17, 133–139.
Xu, G.-J., Natalini, P., Suda, H., Tsolas, O., Dzugaj, A., Sun, S. C., Pontremoli, S., & Horecker, B. L. (1982) Arch. Biochem. Biophys. 214, 688–694.
Zimmermann, G., Kelly, G. J., & Latzko, E. (1976) Eur. J. Biochem. 70, 361–367.
Zimmermann, G., Kelly, G. J., & Latzko, E. (1978) J. Biol. Chem. 253, 5952–5956.

Bovine Cardiac Troponin T: Amino Acid Sequences of the Two Isoforms†

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ABSTRACT: Troponin T (TnT) is the tropomyosin-binding subunit of troponin, the thin filament regulatory complex that confers calcium sensitivity to striated muscle contraction and actomyosin ATPase activity. Bovine cardiac muscle contains two isoforms (TnT-1 and TnT-2) of TnT that differ in sequence near their amino termini. Thin filaments containing TnT-2 require less calcium to activate the MgATPase rate of myosin than do thin filaments containing TnT-1. Using whole troponin T purified from adult bovine cardiac muscle, we have determined the complete amino acid sequence of the larger, more abundant isoform TnT-1. We confirmed that sequence differences between TnT-1 and TnT-2 are confined to the amino-terminal regions and found that TnT-1 makes up approximately 75% of the total troponin T isolated. Partial sequencing of the separated isoforms showed that the difference between them is due solely to residues 15–19 (Glu-Ala-Ala-Glu-Glu) of TnT-1 being absent from TnT-2. The deleted segment may correspond to the product of exon 4 of the chicken cardiac TnT gene [Cooper, T. A., & Ordahl, C. P. (1985) J. Biol. Chem. 260, 11140–11148]. Exon 5, which is developmentally regulated in the chicken, is not expressed in either TnT-1 or TnT-2. TnT-1 contains 284 amino acid residues and has a M_r of 33 808, while TnT-2 contains 279 amino acid residues and has a M_r of 33 279. Bovine cardiac TnT contains the only known thiol group in any isolated TnT (Cys-39 of TnT-1, Cys-34 of TnT-2). Comparison of bovine, rabbit, and chicken cardiac TnT sequences shows near identity of the amino-terminal 13 amino acid residues (exons 2 and 3 of the chicken cardiac gene), many differences in the following 60 residues (exons 4–8), and great similarity in the C-terminal 230 residues (exons 9–18).

The interaction of actin and myosin that occurs during muscle contraction is regulated by changes in intracellular Ca^{2+} concentration. In vertebrate striated muscles the dominant regulatory system involves binding of Ca^{2+} to troponin in the thin filaments. Troponin is a complex of three different protein subunits: TnC binds Ca^{2+}, TnI binds to actin and inhibits actin–myosin interaction, and TnT binds to tropomyosin. The amino acid sequences of all three rabbit fast skeletal muscle troponin subunits have been determined (Wilkinson & Grand, 1974; Collins et al., 1973, 1977; Pearlstone et al., 1976, 1977), and these proteins have served as the models for extensive structure–function studies carried out in several laboratories [see Leavis and Gergely (1984) for review]. Of particular interest for the present study is the manner in which TnT interacts with tropomyosin. The state of our knowledge in this area was recently summarized by White et al. (1987), who have carried out X-ray diffraction studies of the rabbit skeletal muscle troponin–tropomyosin complex. Their results are consistent with biochemical studies, which suggested a two-site model for the attachment of TnT to tropomyosin. TnT is very asymmetric and binds along the length of the carboxyl-terminal third of the tropomyosin molecule in an antiparallel fashion. The amino terminus (residues 1–70) of TnT binds to the carboxyl terminus of tropomyosin and also spans the head-to-tail junction between adjacent tropomyosin molecules in the thin filament, interacting with a small amino-terminal region of the adjacent tropomyosin molecule. This interaction of tropomyosin and the amino-terminal region of TnT forms an invariant, Ca^{2+}-insensitive linkage that is important in maintaining the cooperativity of thin filament proteins. Residues 71–158 of TnT are highly α-helical and extend toward the middle of the tropomyosin molecule, probably forming electrostatic interactions with tropomyosin. The carboxyl-terminal region of TnT binds near residues 150–180 of tropomyosin, ~20 nm away from the head-to-tail junction. In the whole troponin complex, the carboxyl-terminal region of TnT also binds TnC and TnI, and the linkage between TnT and tropomyosin becomes Ca^{2+} sensitive. Binding of Ca^{2+} to TnC apparently breaks this TnT–tropomyosin link and allows the tropomyosin

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to move to a new position on the thin filament, so that the actin–myosin interaction needed for muscle contraction can take place.

Little information is available regarding structural and functional domains of cardiac troponin T. For the relatively few structure–function studies that have been done, bovine cardiac muscle has most frequently been used as the tissue source. In order to carry out additional experiments in this area, the bovine cardiac troponin subunit sequences are needed, but only the sequence of TnC (Van Eerd & Takahashi, 1975) was available. We therefore determined the sequences of bovine cardiac TnI (Leszyk, Dumaswala, Potter, and Collins, unpublished results) and TnT (this report). An additional motivation for sequencing bovine cardiac troponin T was to characterize the differences between its two isoforms (Gusev et al., 1983), which vary in sequence near their amino termini (Risnik et al., 1985). Different calcium concentrations are needed to activate the myosin subfragment 1 MgATPase rate, depending upon which TnT isoform is present in the thin filament (Tobacman, 1986; Tobacman & Lee, 1987). Finally, the presence of a unique, single cysteine residue near the amino terminus of bovine cardiac TnT (Gusev et al., 1983) provides an excellent opportunity for chemical modification studies designed to study the role of TnT in regulating cooperativity of thin filament proteins.

While this work was in progress, the sequence of chicken cardiac TnT, deduced from cDNA and genomic sequences (Cooper & Ordahl, 1985), and the sequence of rabbit cardiac TnT, obtained directly from protein data (Pearlstone et al., 1986), were reported. As with the bovine heart, two variants each of chicken and rabbit cardiac TnT are found, but the details of isoform expression differ. Splicing of exons from the chicken cardiac TnT gene is developmentally regulated: exon 5 is expressed in the embryonic, but not the mature, form of its mRNA. Mature rabbit cardiac muscle contains both a major and a minor isoform, both of which appear to be derived from the same gene by alternative exon splicing. Amino acid residues 13–24 in the major isoform contain eight Glu; in the minor isoform all but one of these Glu are replaced by uncharged residues. It was also noted that Asp and Glu are both present at position 168 of the rabbit cardiac TnT sequence.

Experimental Procedures

Materials. All reagents were of the highest grade commercially available. Sequencer reagents were purchased from Applied Biosystems. PTH[1] amino acid standards were obtained from either Pierce or Applied Biosystems. Amino acid standards, phenyl isothiocyanate (for nonsequencer use), constant-boiling HCl, CNBr, and urea were purchased from Pierce. TPCK-treated trypsin, α-chymotrypsin, and pepsin were obtained from Worthington. SAP was obtained from Boehringer Mannheim. HPLC-grade water, TFA, methanol and β-mercaptoethanol were obtained from Fisher. HPLC-grade acetonitrile was obtained from Burdick & Jackson. Triethylamine was purchased from Aldrich, and iodoacetic acid was from Sigma. Sephadex was purchased from Pharmacia. All other chemicals were obtained from Fisher.

Preparative HPLC. Preparative HPLC was performed on one of two systems. The first system contained a Waters U6K injector, two Waters M510 pumps, a Waters M680 controller, a Waters M480 variable-wavelength absorbance detector, and a Linear dual-channel recorder. The second system contained a Glenco SV-3 injector, a Waters M6000A pump, a Waters M45 pump, a Waters M660 gradient maker, a Gilson Holochrome variable-wavelength absorbance monitor equipped with an HPLC flow cell, and a Linear dual-channel recorder. Equivalent results were obtained with both systems. All separations were carried out by linear gradient elution on a 4.6 mm × 25 cm Vydac 218TP54 reverse-phase column. All of the separations except the chymotryptic digest of CB1 were carried out in the following solvent system. Solvent A was 0.1% TFA in acetonitrile–water (5:95 v/v), and solvent B was 0.1% TFA in acetonitrile–water (95:5 v/v). The chymotryptic digests of CB1 were separated in the following buffer system. Solvent A was 20 mM KH_2PO_4, pH 6.5, and solvent B was acetonitrile–A (60:40 v/v). The flow rates were usually 1.00 mL/min, and the absorbance at 220 nm was monitored. Peptide mixtures were either applied directly to the column or dissolved in a minimum volume of 70% of formic acid. Peptide peaks were collected manually and used directly for amino acid analysis and sequencing.

Amino Acid Compositions and Sequence Analysis. Amino acid compositions were determined by the method of Heinrikson and Meredith (1984). Phenylthiocarbamylated acid hydrolysates of samples were prepared with the Waters PICO-TAG work station. PTC amino acids were analyzed by reverse-phase HPLC on a Waters PICO-TAG column, with the gradient elution system recommended by the manufacturer. Amino acid sequences were determined on an Applied Biosystems Model 470A gas-phase protein sequencer as described by Hewick et al. (1981). PTH amino acids obtained from the sequencer were also analyzed by reverse-phase HPLC, with a Waters Nova-Pak column and the gradient elution system described in Waters Associates Applications Brief M3500. The recovery of PTH amino acids at each cycle was measured quantitatively. PTH-Ser and PTH-Thr were usually obtained in low yields and sometimes not detectable at all; however, they could always be identified by the appearance of breakdown products, which absorbed at 313 nm. PTH-Arg and PTH-His were also often recovered in low yields. A Waters HPLC system including two M510 pumps, a M721 system controller, a WISP 710A autoinjector, a temperature control module, a M440 dual-channel absorbance detector, and a M730 integrative recorder was used for both PTH (sequence) and PTC (composition) amino acid analyses. The detector was set at 254 nm to measure PTC amino acids and the sum of 254 nm (to measure quantitatively PTH amino acids) and 313 nm (for qualitative detection of breakdown products of PTH-Ser and PTH-Thr). Protein structure predictions were done with the aid of the intelligenetics PC/GENE programs SOAP (Kyte & Doolittle, 1982) and GARNIER (Garnier et al., 1978).

Preparation of Protein and Peptide Samples. The large preparation of bovine cardiac TnT that contained both isoforms in a 3:1 (TnT-1:TnT-2) molar ratio was prepared as described by Potter (1982). The purified isoforms TnT-1 and TnT-2 were prepared as described by Gusev et al. (1983) and by Tobacman (1986). Equivalent results were obtained with both methods of isoform purification.

S-Carboxymethylation. A 100-mg preparation of bovine cardiac TnT that contained both isoforms was dissolved in alkylation buffer (50 mM Tris-HCl, 6 M urea, 1 mM dithiothreitol, pH 8). The solution was bubbled with N_2 with constant stirring. The pH was maintained at 8 throughout the entire procedure. After 30 min, 251 μL of β-mercapto-

[1] Abbreviations: PIR, protein identification resource; CNBr, cyanogen bromide; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; HPLC, high-performance liquid chromatography; TPCK, *N*-tosylphenylalanyl chloromethyl ketone; SAP, *Staphylococcus aureus* V8 protease; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

ethanol was added. After another 20 min, 657 mg of iodoacetic acid (dissolved in 1.0 mL of alkylation buffer) was added slowly over a 15-min period. About 10 min after the pH stabilized, an additional 251 μL of β-mercaptoethanol was added. The sample was then immediately desalted on a 5 cm × 55 cm Sephadex G-25 column run in 25% acetic acid.

For S-carboxymethylation of the separated isoforms, 12.5 mg of TnT-1 and 10.0 mg of TnT-2 were each dissolved in 5 mL of alkylation buffer (8 M urea, 1 mM EDTA, 5 mM Tris-HCl, pH 8). After 30 min of bubbling N_2 through the solution and maintaining the pH at 8, a 1000-fold molar excess of β-mercaptoethanol over SH groups was added. After 30 min more, an 1100-fold molar excess over SH groups of iodoacetic acid was added over a 15-min period with strict control of pH. N_2 bubbling was continued for 10 min, and then an additional 1000-fold molar excess of β-mercaptoethanol was added. The samples were immediately desalted on a 1 cm × 27 cm Sephadex G-25 column in 25% acetic acid.

CNBr Digestion. Seventy-five milligrams of carboxymethylated TnT (which contained both isoforms) was dissolved in 5 mL of 70% formic acid. Then, 500 mg of CNBr (freshly dissolved in 500 μL of water) was added. The digest was stirred in a closed screw-cap tube for 20 h at room temperature. The digest was stopped by dilution to 60 mL with water and drying on a rotary evaporator. The resulting peptides were separated on a 2.7 cm × 195 cm Sephadex G-50 (superfine) column equilibrated with 25% acetic acid.

For digesting separated TnT-1 and TnT-2, 150 nmol of each isoform was dissolved in 300 μL of 70% formic acid. A 50-fold molar excess over methionine of CNBr (freshly dissolved in 70% formic acid) was added. The reaction took place in a sealed tube for 19 h at room temperature with constant stirring. The reaction mixture was then diluted to 3 mL with water and dried under N_2. The samples were reconstituted with 200 μL of 70% formic acid and applied directly to a Vydac 218TP54 reverse-phase column.

Chymotryptic Digestion of CB1. Fifty nanomoles of the CB1 fraction obtained from the preparation of TnT that contained both isoforms was dissolved in 800 μL of digest buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.1). To this was added 20 μL of a chymotryptic solution (1 mg/1 mL, freshly dissolved in water). The digest was incubated at 37 °C for 19 h and subsequently stopped with 200 μL of glacial acetic acid. The digestion mixture was separated by reverse-phase HPLC with the KH_2PO_4 buffer system.

Thirty nanomoles of the CB1 fraction of each of the TnT isoforms was dissolved in 300 μL of digest buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.1). Thirty microliters of chymotrypsin (freshly dissolved in digest buffer to a concentration of 1 mg/1 mL) was added. The digest mixture was incubated at 37 °C for 23 h. The digest was subsequently stopped by the addition of 100 μL of glacial acetic acid. The mixture was separated by reverse-phase HPLC with the KH_2PO_4 buffer system.

SAP Digestion of CB1. Fifty nanomoles of CB1 (which contained both isoforms) was dissolved in 200 μL of digest buffer (0.1 M NH_4HCO_3, 2 mM EDTA, pH 7.95). To this was added 20 μL of a 1 mg/1 mL solution (freshly dissolved in water) of SAP. The digest was incubated at 37 °C for 2.5 h and then stopped by the addition of 100 μL of 88% formic acid. The peptides were separated by reverse-phase HPLC in the TFA solvent system.

Tryptic Digestion of CB1. Fifty nanomoles of CB1 (which contained both isoforms) was dissolved in 200 μL of digest buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.1). To this was added 20 μL of a 1 mg/1 mL solution of TPCK-trypsin. The digest was incubated at 37 °C for 24 h. The digestion mixture was applied directly to the reverse-phase column in the TFA solvent system.

Peptic Digestion of CB3. Thirty-nine nanomoles of CB3 was dissolved in 200 μL of a 5% formic acid solution. To this was added 20 mL of pepsin (freshly dissolved in 5% formic acid to a concentration of 1 mg/1 mL). The digest mixture was stirred at room temperature for 3 h and subsequently applied directly to the reverse-phase column in the TFA solvent system.

SAP Digestion of CB4. Twenty-eight nanomoles of CB4 was dissolved in 400 μL of digest buffer (0.1 M NH_4HCO_3, 2 mM EDTA, pH 7.8) to form a cloudy suspension. To this was added 40 μL of a SAP solution (1 mg/1 mL). The digest occurred for 6 h at 37 °C to form a clear solution. The digest was stopped with 100 μL of 88% formic acid and subsequently applied to the reverse-phase column in the TFA solvent system.

Chymotryptic Digestion of CB4. Twenty-eight nanomoles of CB4 was dissolved in 400 μL of digest buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.0) to form a cloudy suspension. To this was added 100 μL of α-chymotrypsin (1 mg/1 mL). The digest occurred for 21 h at 37 °C to form a clear solution. The mixture was applied directly to the reverse-phase column in the TFA solvent system.

SAP Digestion of TnT. Five milligrams of TnT (containing both isoforms) was dissolved in 1 mL of digest buffer (0.1 M NH_4HCO_3, 2 mM EDTA, pH 8.3) to form a cloudy suspension. Then, 100 μL of a SAP solution (1 mg/1 mL, freshly dissolved in water) was added, and the digest was incubated at 37 °C for 4 h to form a clear solution. The digestion mixture was applied directly to the reverse-phase column in the TFA solvent system.

Tryptic Digestion of TnT. Five milligrams of TnT (containing both isoforms) was dissolved in 1.44 mL of digest buffer (0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.0) to form a cloudy suspension. Then, 60 μL of a TPCK-trypsin solution (1 mg/1 mL) was added, and the digest was incubated at 37 °C for 16 h to form a clear solution. The digestion mixture was subsequently applied directly to the reverse-phase column in the TFA solvent system.

Results and Discussion

The strategy and results of our sequence determination of bovine cardiac TnT are summarized in Figure 1. For the sake of clarity, Figure 1 shows only the peptides that are needed to establish the complete sequence of the larger, more abundant isoform TnT-1. During the course of this work we characterized many other peptides and obtained a plethora of redundant information to support this sequence. The sequence of TnT-1 was determined from whole adult bovine cardiac TnT. During the course of our studies (see below) we determined that this preparation, which was recovered as a single peak after two steps of ion-exchange chromatography (Potter, 1982), was a mixture of 75% TnT-1 and 25% TnT-2. The relative amounts of TnT-1 and TnT-2 may, however, be different in the intact bovine heart, since we cannot rule out the possibility of selective enrichment of one isoform during the course of purification.

As with most other contractile proteins, the amino termini of both TnT-1 and TnT-2 were blocked, and so no information could be obtained from direct sequence analysis. We began by digesting 75 mg (2300 nmol) of whole TnT with CNBr. The digest was fractionated by size-exclusion chromatography on Sephadex G-50, yielding pure CB1 and CB2 and a mixture of CB3 and CB4. CB3 and CB4 were then separated by

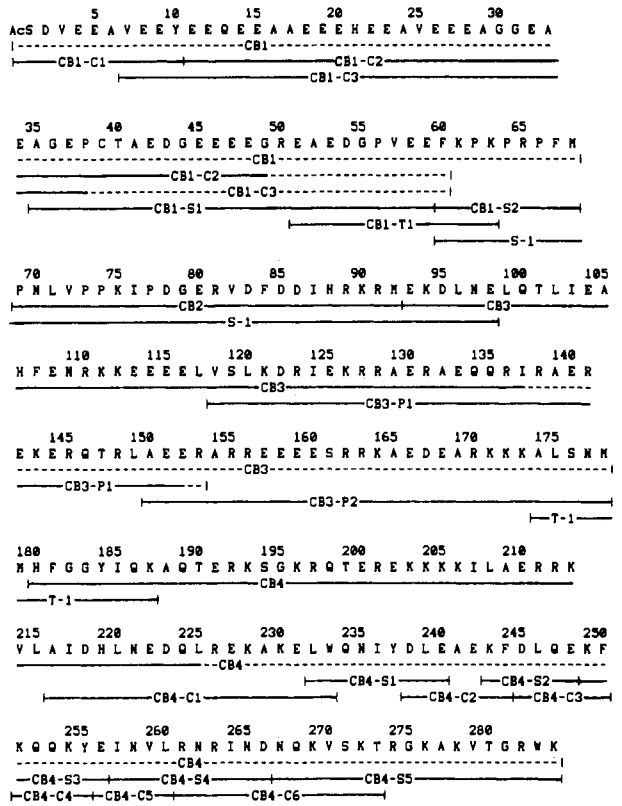

FIGURE 1: Amino acid sequence of TnT-1, the larger, more abundant isoform of troponin T purified from adult bovine cardiac muscle. The figure shows peptides obtained by cleavage with CNBr (CB), trypsin (T), chymotrypsin (C), pepsin (P), and *S. aureus* V8 protease (S). Solid lines indicate residues identified by sequence analysis. Only the results needed to unequivocally establish the sequence are shown. The smaller isoform, TnT-2, differs only by the deletion of residues 15–19.

ion-exchange chromatography on SP-Sephadex. In later experiments, we found that purification of all four CNBr peptides could also be achieved by reverse-phase HPLC. Sequence analysis of each peptide confirmed that CB1 was blocked, gave a complete sequence of CB2, and yielded partial sequences of CB3 and CB4. Next, CB1, CB3, and CB4 were further broken down by several types of proteolytic cleavage, and the resulting subpeptides were purified by HPLC and characterized. Sequence determination of the amino-terminal decapeptide CB1-C1, including the identification of its acetyl blocking group, was accomplished by mass spectrometric analysis. The terminal sequences, residues 1–5 and 283–284, agree with previous observations by Gusev et al. (1983). The weakest part of our sequence evidence concerns the single-residue overlap (at Glu-256) of CB4-S3 and CB4-S4 by CB4-C5. However, we are confident that this assignment is correct, since it is consistent with all the other evidence for the sequence of CB4. Furthermore, the sequence of residues 254–263 is completely conserved, with no insertions or deletions, in both chicken cardiac and rabbit cardiac TnT (see Figure 3). Various proteolytic digests of unfractionated TnT were prepared in order to obtain methionine-containing overlap peptides. Two peptides, purified from different digests by reverse-phase HPLC, were sequenced to yield the necessary information. Peptide S-1 established the alignment CB1-CB2-CB3, and peptide T-1 established CB3-Met-CB4.

A careful examination of all our sequence analyses showed that CB1-C2 (residues 11–60) and CB1-C3 (residues 7–60) were the only peptides that exhibited heterogeneity. The amino-terminal sequences of these peptides were homogeneous, but heterogeneity appeared beginning with Ala-16. This is consistent with previous findings (Risnik et al., 1985) that sequence differences between TnT-1 and TnT-2 are confined to the amino-terminal portion of the molecules, more specifically to the segments that precede the single cysteine residue (residue 39 in Figure 1). For both CB1-C2 and CB1-C3, the molar ratio (as determined by quantitative analysis of PTH amino acids) between the major (as shown in Figure 1) and minor sequences was 3:1.

In order to precisely identify the sequence differences between TnT-1 and TnT-2, we conducted partial sequence studies on the separated isoforms. We obtained essentially identical results with isoforms that were prepared in the USSR (by N.B.G. and A.D.V.) and in the U.S. (by L.S.T.). The CNBr peptides of each isoform were purified by HPLC. Amino acid analyses of CB1 to CB4, complete sequence analyses of CB2, and partial sequence analyses of CB3 and CB4 failed to reveal any differences between TnT-1 and TnT-2. To further characterize the amino-terminal regions, a series of chymotryptic peptides of CB1 were purified and characterized. CB1-C2 was the only peptide that showed isoform-related differences in amino acid composition. This is not surprising, since sequence analysis of CB1-C2 from unfractionated TnT indicated heterogeneity. (CB1-C3 was not obtained from chymotryptic digests of the individual isoforms.) CB1-C2 from TnT-2 had a somewhat lower content of glutamic acid and alanine than CB1-C2 from TnT-1.

Sequence analysis of CB1-C2 from TnT-1 yielded a sequence identical with that of residues 11–40 in Figure 1. The corresponding region of CB1-C2 from TnT-2 was also sequenced and was found to be shorter by five residues. Specifically, the TnT-1 segment Glu-Glu-Ala-Ala-Glu-Glu-Glu, which occurs between Gln-13 and His-21, had been shortened in TnT-2 to Glu-Glu. Since it had previously been shown (Risnik et al., 1985) that the sequence difference between TnT-1 and TnT-2 is located in the amino-terminal region preceding the single cysteine residue (Cys-39 in Figure 1), we conclude that the deletion of two Ala and three Glu in this region accounts for the entire difference between the two isoforms. This conclusion is further supported by the sequence analyses of CB1-C2 and CB1-C3 from unfractionated bovine cardiac TnT. While the major sequence of each of these heterogeneous peptides was identical with that of TnT-1 (as shown in Figure 1), the minor sequence was fully consistent with the presence of the corresponding peptide from TnT-2.

The existence of isoforms of bovine cardiac TnT was first noticed by Gusev et al. (1983), who observed multiple peaks on ion-exchange chromatography of bovine cardiac troponin on DEAE-cellulose in the presence of 8 M urea. Although the multiple peaks were due in large measure to aggregation and differences in phosphorylation, two isoforms of different size were also observed. Later, three isoforms of bovine cardiac TnT, with different charges but similar molecular weights, were observed by Cachia et al. (1985). These three were separated by ion-exchange HPLC but not characterized with regard to differences in sequence or phosphorylation state. Wilkinson and Taylor (1986) recognized two isoforms of bovine cardiac TnT by two-dimensional gel electrophoresis, with no difference in the expression of these isoforms between atria and ventricles. In a more detailed study, Risnik et al. (1985) determined that the molecular weights of TnT-1 and TnT-2 were 33 000 and 31 500, respectively, and that TnT-1 "has an additional peptide enriched with glutamic acid and alanine that is inserted between the N-terminal pentapeptide and the cysteine". Although our complete sequences of TnT-1 and TnT-2 yield somewhat different molecular weights (33 808 and

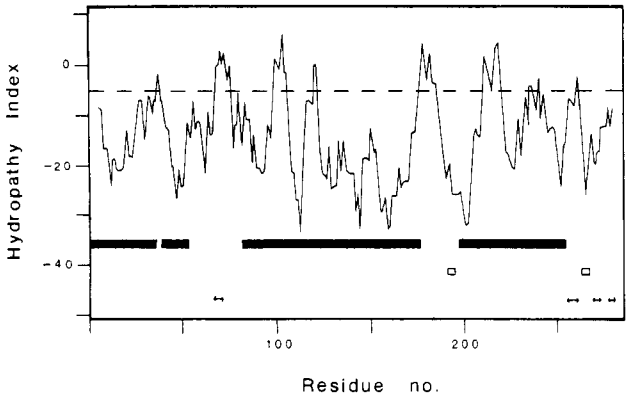

FIGURE 2: Hydropathy plot of TnT-1, showing the locations of predicted α-helices (heavy bars), extended β-strands (arrows), and turns (open bars). The method of Kyte and Doolittle (1982) was used, with a span setting of nine residues. Values above the dashed line indicate particularly hydrophobic regions. Secondary structure was predicted by the method of Garnier et al. (1978).

33 279, respectively), our sequence data are otherwise in complete agreement with the findings of Risnik et al. (1985).

Since TnT is an asymmetric protein that interacts extensively with tropomyosin and also binds TnC and TnI, it would be expected to have a relatively high proportion of its amino acid side chains on the surface. This is supported by the hydropathy plot (Figure 2) of bovine cardiac TnT-1, which shows only a few short, moderately hydrophobic segments that may participate in the formation of a rather limited hydrophobic core. All of the nine proline residues are located in residues 38–77, with seven of these in the short span of residues 62–77. This relatively high concentration of proline residues reduces the likelihood of α-helical or any other type of regular structure in this region of the molecule. Secondary structure prediction (Figure 2) indicates that nearly three-fourths of the molecule may be in the α-helical conformation. A search for amphipathic helices (with a polar face and a nonpolar face) was carried out. Such helices, which may be important in forming the hydrophobic core of a protein, can be detected by a 4-3-4 linear distribution of nonpolar amino acid residues. Using the "helical wheel" technique (Schiffer & Edmundson, 1967), we found that this type of secondary structure may be formed in the carboxyl-terminal region at residues 225–264 of bovine cardiac TnT-1 (and corresponding regions of the other TnT sequences). With the exceptions of Lys-243 and Gln-253, nonpolar residues are found in all the expected positions in this region.

About half of the total residues of bovine cardiac TnT are charged at neutral pH. Residues 1–59 (in TnT-1) constitute a very acidic segment, with a net charge of −29. The carboxyl-terminal 36 residues are strongly basic, with a net charge of +10. The remainder of the charged groups are distributed fairly evenly. Residues 108–172 of bovine cardiac TnT-1 are of special interest. Their distribution of acidic and basic residues is such that the predicted helix in this region could be stabilized by an extensive network of intrahelical salt bridges. This region corresponds to a tropomyosin-binding, highly α-helical (Pearlstone & Smillie, 1977) segment in rabbit skeletal TnT which Sundaralingam et al. (1985) have suggested may form a long central helix of approximately 18 turns, stabilized by intrahelical salt bridges of the type seen in the crystal structure of troponin C. Comparison with the corresponding sequences of rabbit skeletal and bovine cardiac muscle TnT shows a remarkable conservation of all positively and negatively charged residues over the entire span of this putative long helix. In fact, with only two exceptions in the chicken cardiac protein, this distribution of charges is maintained in all known TnT sequences (see Figure 3). Earlier, Parry (1981) noticed (in rabbit skeletal muscle TnT) a significant periodicity of 8.7 residues in the linear distribution of both acidic and basic residues in this segment and suggested that many of the charged groups in this region of TnT may be involved in intrachain ionic interactions. Analysis of the tropomyosin sequence revealed no complementary periodicity of 8.7 residues in the linear disposition of either acidic or basic residues. Furthermore, tropomyosin contains no region of equivalent length which contains such a high content of charged residues.

The amino-terminal region of TnT, which may be important in modulating the cooperative behavior of thin filament proteins (Tobacman, 1986; Tobacman & Lee, 1987), has a high potential for variability due to alternative gene splicing (Wilkinson, 1984; Medford et al., 1984). Tissue-specific and developmentally regulated alternative splicing of split codons at the ends of exons 4–8 of the rat skeletal TnT gene, together with mutually exclusive alternative splicing of exons 16 and 16a, may lead to the expression of as many as 64 different isoforms (Breitbart et al., 1985; Breitbart & Nadal-Ginard, 1986). In keeping with this, apparent multiple isoforms of TnT have been observed by two-dimensional electrophoreses of chicken skeletal muscle (Bird et al., 1985; Imai et al., 1986; Abe et al., 1986). On the other hand, a more detailed characterization of rabbit skeletal muscle TnT showed that only a few of the potential isoforms are actually expressed in this animal (Briggs et al., 1984, 1987). It is noteworthy that, although a very similar exon structure, with equal potential for alternative splicing, is present in both the rat skeletal and chicken cardiac TnT genes (see Figure 3), only two isoforms, resulting from developmentally regulated alternate splicing of a single primary RNA transcript, are actually expressed in the chicken.

The expression of bovine, chicken, and rabbit cardiac TnT isoforms seems to be controlled by different mechanisms. In chicken cardiac muscle, the larger isoform occurs only in the embryo (Cooper & Ordahl, 1985), while in adult bovine heart the larger isoform predominates. In adult rabbit cardiac muscle the two isoforms do not differ in size, although the replacement of seven Glu by neutral residues (Pearlstone et al., 1986) is certainly a significant chemical change. Developmental regulation has so far been demonstrated only in the chicken heart, where exon 5 is expressed in the embryo but spliced out of the mRNA in the adult. The adult bovine and rabbit cardiac TnTs are also missing the decapeptide product of exon 5 but, unlike the chicken, show further variation. The difference between bovine cardiac TnT-1 and bovine cardiac TnT-2 may be attributed to the deletion of residues 15–19 (Glu-Ala-Ala-Glu-Glu) from TnT-1, corresponding to exon 4 of the chicken cardiac gene. The sequence variability between the two isoforms of rabbit cardiac TnT also occurs in this region. Alternative splicing of exon 4 may be an additional source of variability that occurs in adult mammalian, but not avian, cardiac muscle. Investigations of cardiac TnT isoforms in other species are only beginning. How, whether, and under what circumstances cardiac TnT isoform expression may be specifically regulated in species other than chicken have yet to be established.

An overall comparison of the major isoforms of bovine (284 residues), chicken (302 residues), and rabbit (276 residues) cardiac TnTs shows that the three proteins are very similar. With the exception of the hypervariable amino-terminal regions, the sequences are about 90% identical. A noteworthy

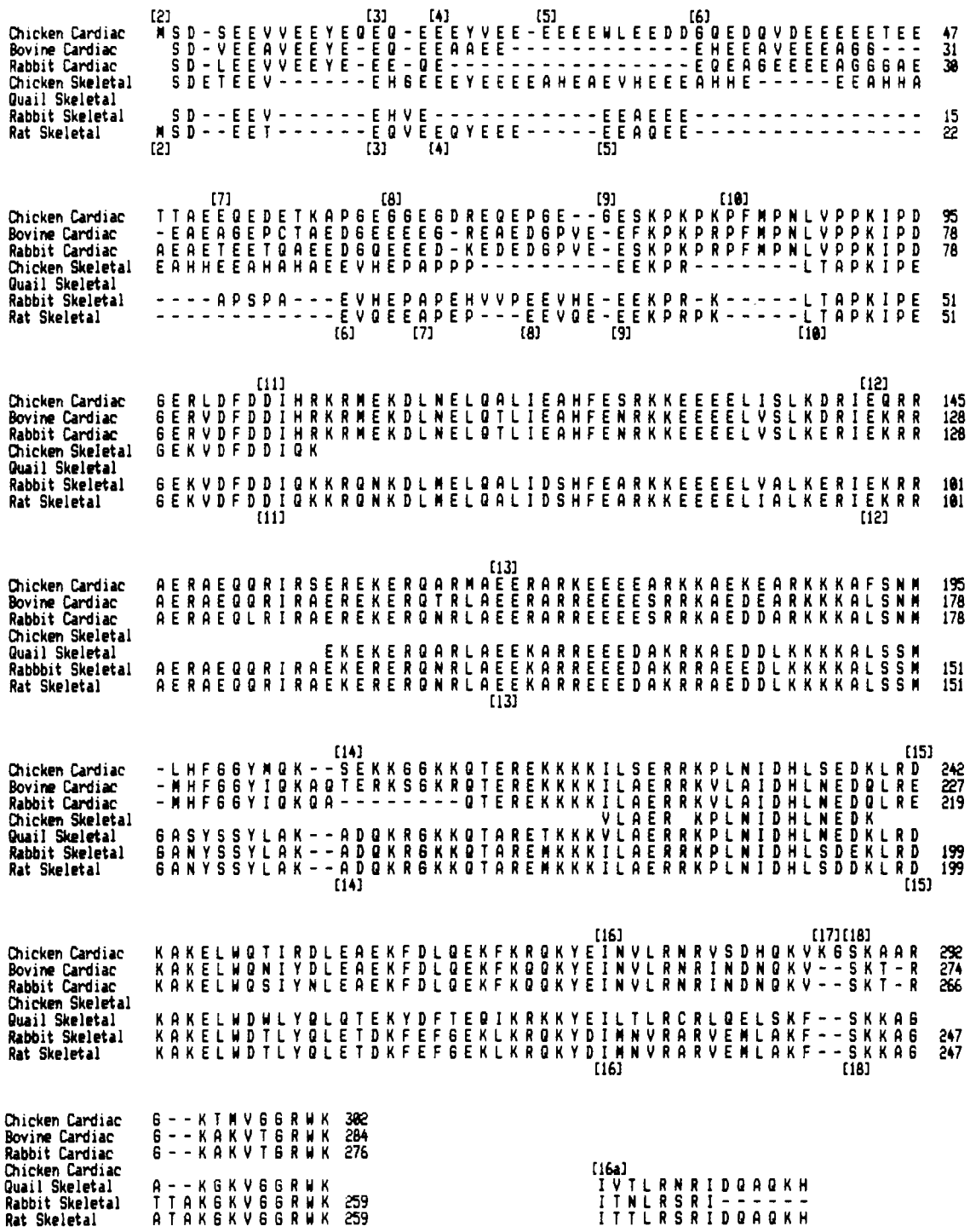

FIGURE 3: Alignment of TnT amino acid sequences. A dash (–) indicates a deletion introduced to maximize sequence similarities. Unsequenced regions are left blank. The last amino acid residue in each line of continuous sequence is numbered. The continuous sequences of the rat skeletal (Medford, et al., 1984; Breitbart & Nadal-Ginard, 1986) and chicken cardiac (Cooper & Ordahl, 1985) proteins were derived from genomic and cDNA sequences. Bracketed numbers aligned with the first amino acid residue translated from each exon indicate the exon numbers and boundaries for these two proteins. The bracketed numbers above each line of sequence are for chicken cardiac TnT, and those below are for rat skeletal TnT. The partial sequence of quail TnT was determined from cDNA (Hastings et al., 1985). Alternative RNA splicing of exons 16 and 16a has been observed in rat (Medford et al., 1984), rabbit (Putney et al., 1983), and quail (Hastings et al., 1985) skeletal muscle TnT as indicated. The continuous rabbit skeletal TnT sequence was determined from the protein (Pearlstone et al., 1976) and in part from cDNA (Putney et al., 1983). The chicken skeletal TnT segments were determined from protein data alone (Wilkinson, 1978; Wilkinson et al., 1984) and represent a composite of three isoforms, which probably arose from alternative RNA splicing of segments corresponding to exons 4 and 6. The complete sequences of the major isoforms of rabbit cardiac TnT (Pearlstone et al., 1986), and bovine cardiac TnT (TnT-1, this work) are derived solely from protein data.

difference is a unique eight-residue deletion in rabbit cardiac TnT that corresponds to the beginning of exon 14. This region of rabbit cardiac TnT is otherwise very similar to all other known cardiac and skeletal TnT sequences. Another curiosity is the dipeptide product of exon 17, which is expressed only in chicken cardiac TnT. This exon is noteworthy for being the smallest reported for any protein (Cooper & Ordahl, 1985).

Both rabbit skeletal and bovine cardiac TnT can be phosphorylated at Ser-1 by an endogenous "troponin T kinase" (Gusev et al., 1980, 1983; Risnik & Gusev, 1984). Two additional moles of phosphate can be incorporated into rabbit skeletal muscle TnT at Ser-149/150 and Ser-156/157 by

phosphorylase kinase (Moir et al., 1977; Risnik et al., 1980). In cardiac TnT, three of these latter four Ser (all except Ser-149) are replaced by nonphosphorylatable residues, leaving only one potential site (e.g., at Ser-176 of bovine cardiac TnT-1). Exhaustive treatment of bovine cardiac TnT with protein kinase C leads to the incorporation of 2 mol of phosphate (Katoh et al., 1983), although the sites of phosphorylation of bovine cardiac TnT by protein kinase C were not investigated. AMP-dependent protein kinase does not phosphorylate bovine cardiac TnT (Katoh et al., 1983). The physiological significance, if any, of the phosphorylation of TnT remains to be established.

While most of the amino acid sequence of TnT is conserved among species and between skeletal and cardiac muscle, it can be seen from Figure 3 that cardiac TnT has an extended amino terminus that is rich in glutamic acid. Even among the three known cardiac TnT sequences, there is considerable diversity in both the length (17–45 residues) and sequences of the extra segments. This extended, acidic region is probably close to the junction between adjacent tropomyosin molecules in the thin filament and may play a role in interactions between tropomyosin and actin (Tobacman, 1986; Tobacman & Lee, 1987; Cooper & Ordahl, 1985; White et al., 1987). The high glutamic acid content of the elongated amino-terminal segments means that cardiac TnTs are acidic, while skeletal TnTs are basic. [See Pearlstone et al. (1986) for further discussion of the similarities and differences between skeletal and cardiac TnT sequences.]

It is clear that much remains to be learned about the relationship of the structure of TnT and its role in regulating interactions among thin filament proteins in both skeletal and cardiac muscles. The availability of the complete sequences of the two major isoforms of bovine cardiac troponin T will be very helpful in planning further biochemical and structural studies. In particular, the presence of Cys-39 (the only known thiol group in a purified TnT molecule) in our bovine cardiac TnT provides an excellent opportunity for cross-linking and other chemical modification studies designed to study the interaction of the amino-terminal region of TnT with tropomyosin and other thin filament proteins.

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Supplementary Material Available

Figures 4–17 showing chromatograms of the peptide separations and Tables I–XII containing amino acid compositions and amino acid sequence data (28 pages). Ordering information is given on any current masthead page.

References

Abe, H., Komiya, T., & Obinata, T. (1986) *Dev. Biol. 118*, 42–51.

Biemann, K., & Scoble, H. A. (1987) *Science (Washington, D.C.) 237*, 992–998.

Bird, I. M., Dhoot, G. K., & Wilkinson, J. M. (1985) *Eur. J. Biochem. 150*, 517–525.

Breitbart, R. E., & Nadal-Ginard, B. (1986) *J. Mol. Biol. 188*, 313–324.

Breitbart, R. E., Nguyen, H. T., Medford, R. M., Destree, A. T., Mahdavi, V., & Nadal-Ginard, B. (1985) *Cell. (Cambridge, Mass.) 41*, 67–82.

Briggs, M. M., Klevit, R. E., & Schachat, F. H. (1984) *J. Biol. Chem. 259*, 10369–10375.

Briggs, M. M., Lin, J. J.-C., & Schachat, F. H. (1987) *J. Muscle Res. Cell Motil. 8*, 1–12.

Cachia, P. J., Van Eyk, J., McCubbin, W. D., Kay, C. M., & Hodges, R. S. (1985) *J. Chromatogr. 343*, 315–328.

Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., & Jackman, N. (1973) *FEBS Lett. 36*, 268–272.

Collins, J. H., Greaser, M. L., Potter, J. D., & Horn, M. J. (1977) *J. Biol. Chem. 252*, 6357–6362.

Cooper, T. A., & Ordahl, C. P. (1985) *J. Biol. Chem. 260*, 11140–11148.

Garnier, J., Osguthorpe, D. J., & Robson, B. (1978) *J. Mol. Biol. 120*, 97–120.

Gusev, N. B., Dobrovolskii, A. B., & Severin, S. E. (1980) *Biochem. J. 189*, 219–226.

Gusev, N. B., Baraskaya, N. V., Verin, A. D., Duzhenkova, I. V., Kuchua, Z. A., & Zheltora, A. D. (1983) *Biochem. J. 213*, 123–129.

Hastings, K. E. M., Bucher, E. A., & Emerson, C. P., Jr. (1985) *J. Biol. Chem. 260*, 13699–13703.

Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65–74.

Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Katoh, N., Wise, B. C., & Kuo, J. F. (1983) *Biochem. J. 209*, 189–195.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235–305.

Medford, R. M., Nguyen, H. T., Destree, A. T., Summers, E., & Nadal-Ginard, B. (1984) *Cell (Cambridge, Mass.) 38*, 409–421.

Moir, A. J. G., Cole, H. A., & Perry, S. V. (1977) *Biochem. J. 161*, 371–382.

Parry, D. A. D. (1981) *J. Mol. Biol. 146*, 259–263.

Pearlstone, J. R., & Smillie, L. B. (1977) *Can. J. Biochem. 55*, 1032–1038.

Pearlstone, J. R., Carpenter, M. R., Johnson, P., & Smillie, L. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1902–1906.

Pearlstone, J. R., Johnson, P., Carpenter, M. R., & Smillie, L. B. (1977) *J. Biol. Chem. 252*, 983–989.

Pearlstone, J. R., Carpenter, M. R., & Smillie, L. B. (1986) *J. Biol. Chem. 261*, 16796–16810.

Peterson, N. O., McConnaughey, W. B., & Elson, E. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5327–5331.

Potter, J. D. (1982) *Methods Enzymol. 85*, 241–263.

Putney, S. D., Herlihy, W. C., & Schimmel, P. (1983) *Nature (London) 302*, 718–721.

Risnik, V. V., & Gusev, N. B. (1984) *Biochim. Biophys. Acta 790*, 108–116.

Risnik, V. V., Dobrovolskii, A. B., Gusev, N. B., & Severin, S. E. (1980) *Biochem. J. 191*, 851–854.

Risnik, V. V., Verin, A. D., & Gusev, N. B. (1985) *Biochem. J. 225*, 549–552.

Schiffer, M., & Edmundson, A. B. (1967) *Biophys. J. 7*, 121–135.

Stone, D., Sodek, J., Johnson, P., & Smillie, L. B. (1974) *Proc. FEBS Meet. 31*, 125–136.

Sundaralingam, M., Drendel, W., & Greaser, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7944–7947.

Tobacman, L. S. (1986) *Biophys. J. 49*, 452a.
Tobacman, L. S., & Lee, R. (1987) *J. Biol. Chem. 262*, 4059–4064.
Van Eerd, J. P., & Takahashi, K. (1975) *Biochem. Biophys. Res. Commun. 64*, 122–127.
White, S. P., Cohen, C., & Phillips, G. N., Jr. (1987) *Nature (London) 325*, 826–828.
Wilkinson, J. M. (1978) *Biochem. J. 169*, 229–238.
Wilkinson, J. M., & Grand, R. J. A. (1975) *Biochem. J. 149*, 493–496.
Wilkinson, J. M., & Taylor, R. D. (1986) *J. Mol. Cell. Cardiol. 18*, 291–297.
Wilkinson, J. M., Moir, A. G., & Waterfield, M. D. (1984) *Eur. J. Biochem. 143*, 47–56.

Cross-Linking of Rabbit Skeletal Muscle Troponin with the Photoactive Reagent 4-Maleimidobenzophenone: Identification of Residues in Troponin I That Are Close to Cysteine-98 of Troponin C†

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ABSTRACT: We have used the sulfhydryl-specific, heterobifunctional, photoactivatable cross-linker 4-maleimidobenzophenone (BPMal) to study the interaction of rabbit skeletal muscle troponin C (TnC) and troponin I (TnI). TnC was specifically labeled at Cys-98 by the maleimide moiety of BPMal, and a binary complex was formed with TnI in the presence of Ca^{2+}. Upon photolysis, covalent cross-links were formed between TnC and TnI [Tao, T., Scheiner, C. J., & Lamkin, M. (1986) *Biochemistry 25*, 7633–7639]. The cross-linked heterodimer was digested with cyanogen bromide, pepsin, and chymotrypsin into progressively smaller cross-linked peptides, which were purified by HPLC and then characterized by amino acid analysis and sequencing. We obtained a fraction from the initial CNBr digest that contained the expected peptide CB9 (residues 84–135) of TnC, cross-linked mainly to CN4 (residues 96–116), the "inhibitory region" of TnI. The peptides CN1 and CN3 of TnI were also detected in this fraction, but their molar ratios (compared to CB9) were only about 0.15 each, compared to 0.60 for CN4. Sequence analyses of fractions obtained after peptic and chymotryptic digests of the cross-linked CNBr fraction confirmed that CB9 and CN4 were the major cross-linked species. Quantitative analysis of sequencer results indicated that the residues in TnI that appeared to be most highly cross-linked to Cys-98 of TnC were Arg-108 and Pro-110, and to a lesser extent Arg-103 and Lys-107. These findings are consistent with previous studies on interactions between TnI and TnC and provide, for the first time, direct information on the identities of proximate amino acids in the two proteins.

The interaction of actin and myosin that occurs during muscle contraction is regulated by changes in intracellular Ca^{2+} concentration. In vertebrate striated muscles the dominant regulatory system involves binding of Ca^{2+} to troponin in the thin filaments. Troponin is a complex of three different protein subunits: TnC[1] binds Ca^{2+}, TnI binds to actin and inhibits actin–myosin interaction, and TnT binds to tropomyosin. The amino acid sequences of all three rabbit fast skeletal muscle troponin subunits have been determined (Wilkinson & Grand, 1975; Collins et al., 1973, 1977; Pearlstone et al., 1977), and these proteins have served as the models for extensive structure–function studies carried out in several laboratories [see Leavis and Gergely (1984) for review]. While much remains to be learned about the three-dimensional structure of rabbit skeletal muscle TnI and TnT, analysis of the TnC sequence (Collins et al., 1973) predicted the locations of four Ca^{2+}-binding sites, designated I–IV going from the amino to the carboxyl terminus of the protein. This prediction was confirmed by the more recently available crystal structures of chicken (Sundaralingam et al., 1985) and turkey (Herzberg & James, 1985) TnCs.

Many studies have focused on the mechanism of the transmission of Ca^{2+}-induced structural changes from one thin filament protein to another (Leavis & Gergely, 1984). A key aspect of this problem is the elucidation of interactions among the thin filament proteins, particularly those among the three troponin components. In the case of the binding of TnC to TnI, considerable information has been obtained from studies of the binding of various proteolytic fragments (Grabarek et al., 1981; Syska et al., 1976), from the reactivities of surface

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[1] Abbreviations: CNBr, cyanogen bromide; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; HPLC, high-performance liquid chromatography; TnC, troponin C; TnI, troponin I; BPMal, 4-maleimidobenzophenone; BP-TnC, TnC labeled with BPMal; BP-TnC*I, cross-linked heterodimer of TnI and BP-TnC; BP-CB9*CN4, cross-linked CNBr peptide from BP-TnC*I.

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