

# Amino Acid Sequence of Bovine Cardiac Troponin I<sup>†</sup>

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Received June 19, 1987; Revised Manuscript Received December 9, 1987

**ABSTRACT:** Troponin I (TnI) is the inhibitory subunit of troponin, the thin filament regulatory complex which confers calcium sensitivity to striated muscle actomyosin ATPase activity. We have determined the amino acid sequence of TnI from adult bovine cardiac muscle. This protein is a single polypeptide chain of 211 amino acids with an acetylated amino terminus, a calculated molecular weight of 23 975, and a net charge of +17 at neutral pH. There was no evidence for heterogeneity of the sequence. Comparison with other available TnI sequences shows an amino-terminal extension of 27–33 residues which is present in cardiac but not skeletal TnI. The remainder of the polypeptide is common to both cardiac and skeletal TnI. In the amino-terminal half of the common polypeptide, only 29% of the residues are invariant in all sequences. The carboxyl-terminal half (residues 124–210) is much more highly conserved, with 66% invariant residues. Bovine cardiac TnI and rabbit cardiac TnI are very similar in sequence: only 12 of 26 residues are identical in the amino-terminal segments, but the remaining residues of the proteins are 97% identical.

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: troponin C (TnC)<sup>1</sup> binds  $Ca^{2+}$ , TnI binds to actin–tropomyosin and inhibits actin–myosin interaction, and TnT binds the troponin complex to tropomyosin (Pearlstone & Smillie, 1983). The amino acid sequences of all three rabbit fast skeletal muscle troponin subunits have been determined (Collins et al., 1973, 1977; Wilkinson & Grand, 1975a; Pearlstone et al., 1976, 1977), and they have served as the basis for extensive structure–function studies, carried out in several laboratories, on the details of the interactions among these proteins [see Leavis and Gergely (1984) and Zot and Potter (1987) for reviews].

Rabbit cardiac muscle and bovine cardiac muscle have most frequently been used as the tissue sources for the relatively few studies which have been carried out on cardiac troponin. The sequences of the rabbit cardiac troponin subunits are known (Grand & Wilkinson, 1976; Wilkinson, 1980; Pearlstone et al., 1986), but important biochemical and physiological studies have also been carried out with bovine cardiac troponin [e.g., see review by Potter et al. (1982)]. In order to correlate experiments which have been done with cardiac troponin from both species, and to carry out additional studies, 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 TnT (Leszyk et al., 1987) and TnI (this report). The avail-

ability of another cardiac TnI sequence also enables us to identify consistent similarities and differences between skeletal and cardiac TnI. The most recent comparison of available TnI sequences (chicken and rabbit fast skeletal, rabbit slow skeletal, and rabbit cardiac muscles) was by Wilkinson and Grand (1978a). With the exception of a confirmatory cDNA-derived sequence for chicken fast skeletal TnI (Nikovits et al., 1986), and an identical cDNA sequence for quail fast skeletal muscle TnI (Baldwin et al., 1985), no new TnI sequences have been reported during the past 10 years.

TnI and TnT occur naturally as phosphoproteins (Cole & Perry, 1975; Solaro et al., 1976; England, 1976; Moir & Perry, 1977; Stull & Buss, 1977; Moir et al., 1980). The functional significance of troponin phosphorylation in muscle contraction is not fully understood. It has been suggested that phosphorylation of troponin may be involved in the speed and/or force of contraction (England, 1975; Solaro et al., 1976; Moir & Perry, 1980; Moir et al., 1980),  $Ca^{2+}$ -dependent regulation by troponin (Holroyde et al., 1979; Robertson et al., 1982; Yamamoto & Ohtsuki, 1982), proteolysis of troponin (Toyo-Oka, 1982), and a long-term modulation of cardiac contractility (Villar-Palasi & Kumon, 1981; Katoh et al., 1983).

Cardiac troponin is phosphorylated, exclusively at a Ser residue near the amino terminus of its TnI subunit, when hearts are perfused with  $\beta$ -adrenergic agonists (Solaro et al., 1976; England, 1976; Moir et al., 1980) or when troponin or troponin–tropomyosin preparations are incubated in vitro with cyclic AMP dependent protein kinase (Cole & Perry, 1975; Moir & Perry, 1977; Stull & Buss, 1977; Blumenthal et al., 1978; Lincoln & Corbin, 1978) or cyclic GMP dependent protein kinase (Blumenthal et al., 1978; Lincoln & Corbin, 1978). This phenomenon does not occur in the skeletal tro-

<sup>†</sup> This work was supported by NIH Grants AR35120 (to J.H.C.) and HL22619-3A (to J.D.P.). The Mass Spectrometry Facility, Massachusetts Institute of Technology, was supported by NIH Grant RR0037 (to Klaus Biemann).

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<sup>1</sup> Abbreviations: TnI, troponin I; TnC, troponin C; TnT, troponin T; CM-TnI, S-carboxymethylated TnI; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PTC, phenylthiocarbonyl; HPLC, high-performance liquid chromatography; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; SAP, *Staphylococcus aureus* V8 protease; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)amino-methane hydrochloride.

ponin complex, undoubtedly because skeletal TnI lacks the extended amino terminus of cardiac TnI (Grand & Wilkinson, 1976). This finding has aroused a great deal of interest, since, in beating hearts, it has been possible to correlate the degree of positive inotropy brought about by  $\beta$ -adrenergic agonists and the degree of cardiac TnI phosphorylation (England, 1975; Solaro et al., 1976). The mechanism of this phenomenon has not been established, but it may be related to a lowering of the  $\text{Ca}^{2+}$  affinity of the single  $\text{Ca}^{2+}$ -specific site (on TnC) in phosphorylated cardiac troponin (Robertson et al., 1982).

Skeletal TnI and cardiac TnI are also phosphorylated by phosphorylase kinase (Perry & Cole, 1974; Moir & Perry, 1980), but this is not likely to be of physiological significance (Moir et al., 1977; Stull, 1980). Of greater potential interest is the action of protein kinase C, which appears to have a greater ability, compared to other protein kinases, to phosphorylate both Ser and Thr residues in cardiac and skeletal TnI and TnT (Katoh et al., 1983; Mazzei & Kuo, 1984), suggesting a potential involvement of this kinase in the regulation of myofibrillar activity by troponin.

#### EXPERIMENTAL PROCEDURES

**Materials.** All reagents were of the highest grade commercially available. Sequencer reagents were purchased from Applied Biosystems. PTH-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,  $\alpha$ -chymotrypsin, and pepsin were obtained from Worthington. SAP and Arg-C protease were obtained from Boehringer Mannheim. HPLC-grade water, TFA, methanol, and  $\beta$ -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 and Bio-Gel from Bio-Rad. 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 Holo-chrome variable-wavelength absorbance monitor equipped with an HPLC flow cell, and a Linear dual-channel recorder. Equivalent results were obtained with both systems. Most separations were carried out by gradient elution on a 4.6 mm  $\times$  25 cm Vydac 218TP54 reverse-phase column. Peptides T-1 through T-8, CB3-C2, and CB3-C4 were purified by using a 3.9 mm  $\times$  30 cm Waters  $\mu$ Bondapak C<sub>18</sub> reverse-phase column. Separations carried out on the Vydac column used 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). Separations carried out on the  $\mu$ Bondapak column used a solvent system in which solvent B was acetonitrile and solvent A was either 20 mM  $\text{HK}_2\text{PO}_4$  (pH 2.2) or 20 mM triethylammonium phosphate (pH 3.5). 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 solvent A or 70% formic acid. Peptide peaks were collected manually and used directly for amino acid analysis and sequencing.

**Amino Acid Compositions and Sequence Analysis.** Most amino acid compositions were determined by the method of

Heinrikson and Meredith (1984). Phenylthiocarbamylated acid hydrolysates of samples were prepared by using the Waters "PICO-TAG" work station. PTC-amino acids were analyzed by reverse-phase HPLC on a Waters "PICO-TAG" column, using the gradient elution system recommended by the manufacturer. Most amino acid sequences were determined by using an Applied Biosystems Model 470A gas-phase protein sequencer as described by Hewick et al. (1981). PTH-amino acids obtained from the sequencer were analyzed by reverse-phase HPLC, using 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 autinjector, 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).

In the early stages of this work, some peptides were analyzed with a Glenco MM-60 amino acid analyzer and sequenced on a Beckman 890-C sequencer, according to procedures described previously (Collins et al., 1983).

**Preparation of Protein.** Bovine cardiac TnI was prepared as described by Potter (1982). For alkylation of thiol groups, 75–100 mg of protein was dissolved in 40 mL of alkylation buffer (50 mM Tris-HCl, 6 M urea, and 1 mM dithiothreitol, pH 8). The solution was bubbled with nitrogen under constant stirring. The pH was maintained at 8 throughout the entire procedure. After 30 min, 583  $\mu$ L of  $\beta$ -mercaptoethanol was added. After another 20 min, 1.7 g of iodoacetic acid (dissolved in a minimum volume of alkylation buffer) was added slowly over a 40-min period. About 10 min after the pH had stabilized at 8, an additional 583  $\mu$ L of  $\beta$ -mercaptoethanol was added. The sample was then immediately desalted on a 5 cm  $\times$  55 cm Sephadex G-25 column run in 25% acetic acid. The resulting protein, CM-TnI, was used as the starting material for all peptide generation.

**Tryptic Digestion of CM-TnI.** A 102-mg portion of CM-TnI was dissolved in 8.0 mL of digest buffer (0.2 M *N*-methylmorpholine acetate, pH 7.9). To this was added 1.72 mg of TPCK-trypsin (freshly dissolved in 1.72 mL of water). Digestion was allowed to proceed for 16 h at 37 °C and then stopped by adding 2.0 mL of glacial acetic acid. The digestion mixture was then applied to a 2.7 cm  $\times$  195 cm Sephadex G-50 (superfine) column equilibrated with 25% acetic acid.

**CNBr Digestion of CM-TnI.** A 62-mg portion of CM-TnI was dissolved in 5 mL of 70% formic acid. Then 350 mg of CNBr (freshly dissolved in 350  $\mu$ L of water) was added. The reaction mixture was stirred overnight in a closed screw-cap tube at room temperature. The digest was stopped by dilution to 60 mL with water and drying on a rotary evaporator. The resulting peptides were dissolved in 2.0 mL of 88% formic acid and applied to a 2.7 cm  $\times$  195 cm Sephadex G-50 (superfine) column equilibrated with 25% acetic acid.

**Tryptic Digestion of CB1.** A 50-nmol portion of CB1 was dissolved in 400  $\mu$ L of digest buffer (0.1 M  $\text{NH}_4\text{HCO}_3$ /0.1 mM  $\text{CaCl}_2$ , pH 8.3). To this was added 10  $\mu$ g of TPCK-

trypsin (freshly dissolved in 10  $\mu$ L of water). Digestion was allowed to proceed for 23 h at 37 °C, with further 10- $\mu$ g portions of TPCK-trypsin added at 30 min and 19 h. Digestion was stopped by adding 170  $\mu$ L of 88% formic acid, and the resulting mixture was applied to HPLC.

**Chymotryptic Digestion of CB1.** A 20-nmol portion of CB1 was dissolved in 200  $\mu$ L of digest buffer (0.1 M  $\text{NH}_4\text{HCO}_3$ /0.1 mM  $\text{CaCl}_2$ , pH 8.3). To this was added 20  $\mu$ g of  $\alpha$ -chymotrypsin (freshly dissolved in 20  $\mu$ L of water). Digestion was allowed to proceed for 22 h at 37 °C. Digestion was stopped by adding 100  $\mu$ L of 70% formic acid, and the resulting mixture was applied to HPLC.

**Peptic Digestion of CB1.** A 20-nmol portion of CB1 was dissolved in 200  $\mu$ L of 5% formic acid. To this was added 25  $\mu$ L of pepsin (freshly dissolved in 5% formic acid to a concentration of 1 mg/1 mL). The digest mixture was stirred at 30 °C for 2 h, another 25  $\mu$ L of pepsin was added, digestion was continued for another hour, and the resulting mixture was applied to HPLC.

**Arg-C Protease Digestion of CB1.** A 20-nmol portion of CB1 was dissolved in 200  $\mu$ L of digest buffer (0.1 M  $\text{NH}_4\text{HCO}_3$ , pH 8.3). To this was added 25  $\mu$ g of Arg-C protease (freshly dissolved in 25  $\mu$ L of water). Digestion was allowed to proceed for 2 h at 37 °C; then another 15  $\mu$ g of Arg-C protease was added. After a total digestion time of 5.5 h, the resulting mixture was applied to HPLC.

**Partial Acid Hydrolysis of CB1.** A 15-nmol portion of CB1 was dissolved in 200  $\mu$ L of 0.03 N HCl and incubated at 105 °C for 8.5 h. The digest was cooled to room temperature and applied to cation-exchange chromatography, using the HPLC apparatus described above. The column used was Bio-Gel TSK SP-5-PW (0.75 cm  $\times$  7.5 cm; obtained from Bio-Rad), equilibrated, and run with a solvent of 5 mM  $\text{KH}_2\text{PO}_4$  (pH 2.75) and a flow rate of 0.8 mL/min. Peptides were eluted with a linear, 40-min gradient from zero to 0.5 M KCl.

**SAP Digestion of CB2.** A 100-nmol portion of CB2 was dissolved in 300  $\mu$ L of digest buffer (4 M urea, 0.1 M  $\text{NH}_4\text{HCO}_3$ , and 2 mM EDTA, pH 7.8). To this was added 20  $\mu$ L of a 1 mg/1 mL solution (freshly dissolved in 0.01%  $\text{NaN}_3$ ) of SAP. Digestion took place at 37 °C for 4 h and then was stopped by adding 107  $\mu$ L of 88% formic acid, and the resulting mixture was applied to HPLC.

**Chymotryptic Digestion of CB3.** A 1870-nmol portion of CB3 was dissolved in 900  $\mu$ L of digest buffer (0.2 M *N*-methylmorpholine acetate, pH 8.0). To this was added 300  $\mu$ g of  $\alpha$ -chymotrypsin (freshly dissolved in 100  $\mu$ L of water). Digestion was allowed to proceed overnight at room temperature, and then the digest was applied directly to a 2 cm  $\times$  192 cm column of Bio-Gel P-4 run in 25% acetic acid.

## RESULTS

The strategy and results of our sequence determination of bovine cardiac TnI are summarized in Figure 1. Only the peptides needed to establish the sequence are shown. As with most contractile proteins, the amino terminus of bovine cardiac TnI was blocked, and no information could be obtained from direct sequence analysis. We began by digesting 62 mg (2600 nmol) of CM-TnI with CNBr. The expected four peptides, CB1-CB4, were purified by size-exclusion chromatography on sephadex G-50. Because of incomplete cleavage at Met-53, we also obtained some CB1-CB2.

CB1, the blocked amino-terminal 54 residues of the protein, was initially cleaved with trypsin, and three peptides (CB1-T2, CB1-T3, and CB1-T4) were purified by HPLC and sequenced. A fourth tryptic peptide, CB1-T1, was blocked and assumed from its composition to be X-(Ala,Asx)-Arg, the amino-ter-

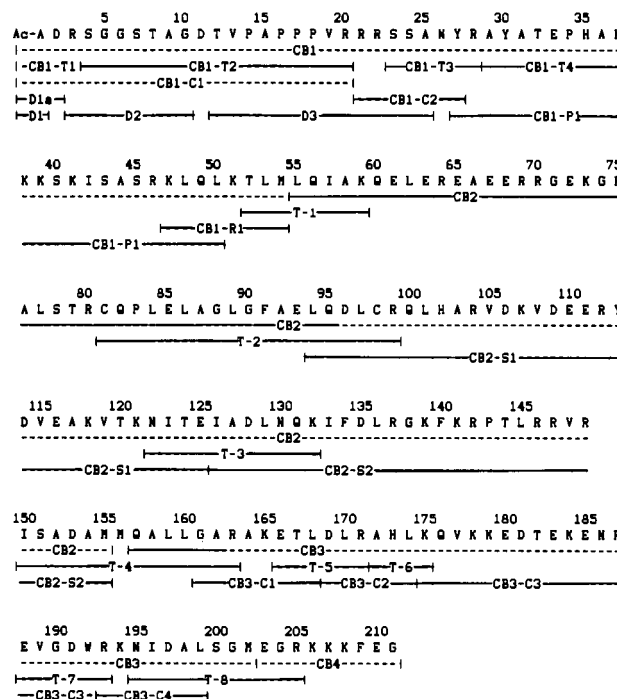


FIGURE 1: Amino acid sequence of bovine cardiac TnI. The figure shows peptides obtained by cleavage with CNBr (CB), trypsin (T), chymotrypsin (C), pepsin (P), *S. aureus* V8 protease (S), Arg-C protease (R), and dilute acid (D). Solid lines indicate residues identified by sequence analysis.

минаl tripeptide of TnI. Due to the specificity of trypsin, it seems safe to presume that Arg is the carboxyl-terminal residue of CB1-T1. Attempts to characterize CB1-T1 by carboxypeptidase digestion or by tandem mass spectrometry (Johnson & Biemann, 1987) were unsuccessful.

HPLC of a chymotryptic digest of CB1 yielded two key peptides, CB1-C1 and CB1-C2. CB1-C2 was sequenced, but CB1-C1 was a blocked peptide with an amino acid composition equivalent to the sum of CB1-T1 and CB1-T2. Mass spectrometry of CB1-C1 established that its total mass was 1950.1 daltons. This mass, together with the other information available, confirms the existence of an amino-terminal acetyl blocking group in TnI and also indicates that CB1-T1 contains an Asp (not Asn) residue. HPLC of a peptic digest of CB1 yielded a peptide, CB1-P1, which gave the sequence of residues 27-50. The carboxyl-terminal sequence of CB1 was obtained from CB1-R1, a peptide purified by HPLC of an Arg-C protease digest.

To complete the sequence of its amino-terminal portion, CB1 was subjected to partial acid hydrolysis under conditions in which peptide bonds on either side of Asp and Asn residues are preferentially cleaved (Tsung & Fraenkel-Conrat, 1965). The resulting peptides were separated by cation-exchange HPLC. Two partially resolved fractions, D1 and D1a, emerged near the breakthrough volume of the column, indicating that they contained no free amino groups. An attempt to sequence D1 was unsuccessful, confirming that its amino terminus was indeed blocked. Amino acid analysis showed that D1 contained mainly Ala, while D1a contained nearly equimolar amounts of Ala and Asp. Neither fraction contained free amino acids, as established by amino acid analysis without prior hydrolysis. These results, combined with the other evidence discussed above, show that D1 is Ac-Ala and D1a is Ac-Ala-Asp. The peptides D3 and D4 were also purified from the partial acid hydrolysate, and their sequences confirmed the expected preferential cleavage at Asp-2, Asp-11, and Asn-26. In addition, the sequence of D3 provided an



weak (Wilkinson & Grand, 1978a). Since the intervening Leu does not occur in our bovine cardiac sequence or in any of the skeletal TnI sequences, we have taken the liberty of removing it from Figure 2.

Bovine cardiac TnI contains a total of 50 basic residues, 33 acidic residues, and 49 residues with bulky, hydrophobic side chains. The hydropathy plot (not shown), prepared by the method of Kyte and Doolittle (1982), reveals a fairly regular pattern of alternating hydrophilic and hydrophobic regions, suggestive of a compact, globular protein with a well-defined hydrophobic core. However, hydrodynamic studies on cardiac TnI (Byers & Kay, 1983) indicate that it is not a typical, compact globular protein. An analysis of the distribution of acidic and basic residues reveals that 25 of the basic residues are located within 4 segments (residues 34–60, 136–152, 170–179, and 204–209) which contain no acidic residues. The remaining 25 basic residues are fairly well interspersed with the 33 acidic residues. The longest segments of concentrated acidic residues uninterrupted by basic residues are located at residues 65–68 (Glu-Ala-Glu-Glu) and 108–111 (Val-Asp-Glu-Glu). It may be significant that by far the longest segment (residues 81–98) which contains no basic residues (and three acidic residues) is that which includes the two Cys residues, located at either end of the segment.

For the purpose of overall comparison, it is convenient to divide the five known TnI sequences into three parts. The amino-terminal extension (residues 1–33), which is present only in the cardiac TnI sequences, extends to the boundary between exons 2 and 3 (Figure 2). By assuming 6 deletions in the rabbit cardiac sequence, we obtain a total of 17 identical residues between rabbit and bovine cardiac TnI in this extension. The remainder of the sequences are common to all five TnI's and may be divided approximately in half at the boundary between exons 6 and 7. In the amino-terminal half of this common region (residues 34–124), only 29% of the residues are invariant in all known sequences. The carboxyl-terminal half (residues 125–211) is much more highly conserved, with 66% invariant residues. This includes four stretches (residues 135–143, 177–185, 189–195, and 200–207) of seven to nine consecutive invariant residues. The greatest variability in the carboxyl-terminal region is found around Asn-186, where deletions of one to three residues have occurred. As noted by Wilkinson and Grand (1978a), this region includes a Pro residue in slow skeletal TnI and may represent a bend or flexible segment where sequence changes can be tolerated without affecting the overall three-dimensional structures of the proteins.

With the exception of their amino-terminal extensions, the sequences of bovine cardiac and rabbit cardiac TnI's are remarkably similar. Residues 37–41 are Lys-Lys-Lys-Ser-Lys in bovine cardiac TnI and Lys-Ser-Lys-Lys-Lys in rabbit cardiac TnI. This slight difference could be due to an error in aligning small tryptic peptides of rabbit cardiac TnI (Grand et al., 1976). Over the span of residues 28–211, the only other differences from bovine cardiac TnI are the substitution in rabbit of Thr for Asn-130, Thr for Ala-162, and Leu for Ala-198.

There are two regions of TnI (residues 33–80 and 130–150) which have been shown to bind to TnC (Syska et al., 1976; Leavis et al., 1978; Grabarek et al., 1981). Residues 33–80 are located in the relatively variable amino-terminal half of TnI and contain only 23% invariant residues. This variability may contribute to functional differences among TnI's in different muscles. It should be kept in mind, however, that the interaction of this region of TnI with the acidic protein TnC

probably involves the highly basic segment encompassed by residues 37–51. Although this segment contains only four invariant residues, most of the basic residues are conserved.

The region of residues 130–150 binds to both TnC and actin and exhibits inhibitory activity similar to that of whole TnI [see review by Leavis and Gergely (1984) and Zot and Potter (1987)]. The most important segment for inhibitory activity appears to be residues 132–147 (Talbot & Hodges, 1979, 1981a), which bind to tropomyosin-actin as well as to TnC (Cachia et al., 1983, 1986). This segment is completely conserved in the known TnI sequences (Figure 2), except for substitutions of similar hydrophobic residues at positions 133 and 134, and the substitution at position 144 of a Pro in skeletal TnI for Thr in cardiac TnI. This Pro/Thr substitution might be expected to induce a functionally important structural difference between skeletal and cardiac TnI. Four reverse turns were predicted in rabbit skeletal muscle TnI by Huang et al. (1974), using the method of Chou and Fasman (1978). One of these turns occurs at residues 143–146 (Pro-Thr-Leu-Arg in cardiac TnI and Pro-Pro-Leu-Arg in skeletal TnI). According to the rules of Chou and Fasman (1978), the effect of substituting Thr for Pro in this sequence would be to reduce the probability of turn formation by a factor of 3. On the other hand, Talbot and Hodges (1981b) showed that the Pro/Thr substitution had no effect on the inhibitory activity of peptides derived from this region of TnI.

Cyclic AMP dependent protein kinase rapidly phosphorylates Ser-23 in the extended amino terminus of cardiac TnI and more slowly phosphorylates Ser-151 in both cardiac and skeletal TnI (Moir et al., 1974; Huang et al., 1974; Moir & Perry, 1977). These residues may play a functionally important role, since, as can be seen in Figure 2, Ser-23 is conserved in cardiac TnI and Ser-151 is invariant in all known TnI sequences. Identification of the sites on TnI which are phosphorylated by protein kinase C and determination of the effects of phosphorylation on the interactions of contractile proteins may yield new insights into the regulatory role played by protein kinase C in the regulation of muscle contraction.

Two of the three Cys residues of rabbit skeletal TnI correspond to the two Cys residues (81 and 98) of bovine cardiac TnI. These two Cys residues are known to be involved in TnI-TnT interactions (Chong & Hodges, 1982; Ingraham & Hodges, 1987) and must be in a reduced state in order for TnI to form functional complexes with the other troponin subunits (Horwitz et al., 1979; Hinckle et al., 1979; Potter et al., 1982). Air oxidation apparently results in the formation of an intrachain disulfide bond between Cys-81 and Cys-98 of bovine cardiac TnI, and one may assume that these two residues are close together in the structure. The possible functional importance of Cys-81 and Cys-98 is further indicated by their conservation in the other known TnI sequences, with the exception that Cys-81 of rabbit slow skeletal TnI has been substituted by an Ile (see Figure 2).

Tryptophan may play an important role in the functioning of TnI. Each of the 5 known TnI sequences contains a single Trp residue, located in the center of an invariant heptapeptide sequence (Val-Gly-Asp-Trp-Arg-Lys-Asn), about 20 residues from the carboxyl terminus of the protein. Although Trp itself is a hydrophobic residue, it is located within a hydrophilic region of the sequence and would be expected to be at or near the surface of the TnI molecule. Wang and Gergely (1986) have observed a change in TnI Trp fluorescence in the rabbit fast skeletal TnC-TnI complex, caused by binding of  $\text{Ca}^{2+}$  to the two  $\text{Ca}^{2+}$ -specific regulatory sites in the amino-terminal half of TnC.

Other residues in TnI which may be of interest for chemical modification studies are the invariant His-102 and Tyr-113, neither of which is located within a region of TnI which is thought to interact with TnC.

It is clear that much remains to be learned about the relationship of the structure of TnI and its role in regulating interactions among thin filament proteins in both skeletal and cardiac muscles. The mechanism by which  $\text{Ca}^{2+}$ -dependent changes in TnC lead to a release of TnI from actin-tropomyosin remains a key unsolved problem in muscle biology. The availability of the sequence of bovine cardiac troponin I will be useful in planning further biochemical and structural studies designed to help solve this problem.

#### ACKNOWLEDGMENTS

We thank Drs. Hubert Scoble and Klaus Biemann of the Mass Spectrometry Facility, Massachusetts Institute of Technology, for the mass spectrometric analysis. We thank Janet Theibert for help with the amino acid and sequence analyses, Karen West for preparation of the figures, and Stephanie Shelly for preparation of the manuscript. We gratefully acknowledge a generous gift of bovine cardiac TnI from Dr. Paul Leavis of the Boston Biomedical Research Institute.

#### SUPPLEMENTARY MATERIAL AVAILABLE

Figures 3–20 showing chromatograms of the peptide separations and Tables I–VII containing amino acid compositions and amino acid sequence data (29 pages). Ordering information is given on any current masthead page.

#### REFERENCES

- Baldwin, A. S., Jr., Kittler, E. L. W., & Emerson, C. P., Jr. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 8080–8084.
- Blomback, B. (1967) *Methods Enzymol.* 11, 398–411.
- Blumenthal, D. K., Stull, J. T., & Gill, G. N. (1978) *J. Biol. Chem.* 253, 334–336.
- Byers, D. M., & Kay, C. M. (1983) *J. Biol. Chem.* 258, 2951–2954.
- Cachia, P. J., Sykes, B. D., & Hodges, R. S. (1983) *Biochemistry* 22, 4145–4152.
- Cachia, P. J., Van Eyk, J., McCubbin, W. D., Kay, C. M., & Hodges, R. S. (1985) *J. Chromatogr.* 343, 315–328.
- Cachia, P. J., Van Eyk, J., Ingraham, R. H., McCubbin, W. D., Kay, C. M., & Hodges, R. S. (1986) *Biochemistry* 25, 3553–3562.
- Chong, P. C. S., & Hodges, R. S. (1981) *J. Biol. Chem.* 256, 5071–5076.
- Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem.* 47, 251–276.
- Cole, H. A., & Perry, S. V. (1975) *Biochem. J.* 149, 525–533.
- 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.
- Collins, J. H., Johnson, J. D., & Szent-Gyorgyi, A. G. (1983) *Biochemistry* 22, 341–345.
- England, P. J. (1975) *FEBS Lett.* 50, 57–60.
- England, P. J. (1976) *Biochem. J.* 160, 295–304.
- Grabarek, Z., Drabikowski, W., Leavis, P. C., Rosenfeld, S. S., & Gergely, J. (1981) *J. Biol. Chem.* 256, 13121–13127.
- Grand, R. J. A., & Wilkinson, J. M. (1976) *Biochem. J.* 159, 633–641.
- Grand, R. J. A., & Wilkinson, J. M. (1977) *Biochem. J.* 167, 183–192.
- Grand, R. J. A., Wilkinson, J. M., & Mole, L. E. (1976) *Biochem. J.* 159, 633–641.
- 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.
- Hincke, M. T., McCubbin, W. D., & Kay, C. M. (1979) *Can. J. Biochem.* 57, 768–775.
- Holroyde, M. J., Howe, E., & Solaro, R. J. (1979) *Biochim. Biophys. Acta* 586, 63–69.
- Horwitz, J., Bullard, B., & Mercola, D. (1979) *J. Biol. Chem.* 254, 350–355.
- Huang, T. S., Bylund, D. B., Stull, J. T., & Krebs, E. G. (1974) *FEBS Lett.* 42, 249–252.
- Ingraham, R. H., & Hodges, R. S. (1987) *Biophys. J.* 51, 326a.
- Johnson, R. S., & Biemann, K. (1987) *Biochemistry* 26, 1209–1214.
- 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.
- Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem.* 253, 5452–5459.
- Leszyk, J., Dumaswala, R., Potter, J. D., Gusev, N. B., Verin, A. D., Tobacman, L. S., & Collins, J. H. (1987) *Biochemistry* 26, 7035–7042.
- Lincoln, T. M., & Corbin, J. D. (1978) *J. Biol. Chem.* 253, 337–339.
- Mazzei, G. J., & Kuo, J. F. (1984) *Biochem. J.* 218, 361–369.
- Moir, A. J. G., & Perry, S. V. (1977) *Biochem. J.* 167, 333–343.
- Moir, A. J. G., & Perry, S. V. (1980) *Biochem. J.* 191, 547–554.
- Moir, A. J. G., Wilkinson, J. M., & Perry, S. V. (1974) *FEBS Lett.* 42, 253–256.
- Moir, A. J. G., Cole, H. A., & Perry, S. V. (1977) *Biochem. J.* 161, 371–382.
- Moir, A. J. G., Solaro, R. J., & Perry, S. V. (1980) *Biochem. J.* 185, 505–513.
- Nikovits, W., Jr., Kuncio, G., & Ordahl, C. P. (1986) *Nucleic Acids Res.* 14, 3377–3390.
- Pearlstone, J. R., & Smillie, L. B. (1983) *J. Biol. Chem.* 258, 2534–2542.
- 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.
- Perry, S. V., & Cole, H. A. (1974) *Biochem. J.* 141, 733–743.
- Potter, J. D. (1982) *Methods Enzymol.* 85, 241–263.
- Potter, J. D., Holroyde, M. J., Robertson, S. P., Solaro, R. J., Kranias, E. G., & Johnson, J. D. (1982) in *Cell and Muscle Motility* (Dowben, R. M., & Shay, J. W., Eds.) Vol. 2, pp 245–255, Plenum, New York.
- Robertson, S. P., Johnson, J. D., Holroyde, M. J., Kranias, E. G., Potter, J. D., & Solaro, R. J. (1982) *J. Biol. Chem.* 257, 260–263.
- Solaro, R. J. (1982) *J. Biol. Chem.* 257, 260–263.
- Solaro, R. J., Moir, A. J. G., & Perry, S. V. (1976) *Nature (London)* 262, 615–617.
- Stull, J. T. (1980) *Adv. Cyclic Nucleotide Res.* 13, 39–43.

- Stull, J. T., & Buss, J. E. (1977) *J. Biol. Chem.* 252, 851-857.
- Syska, H., Perry, S. V., & Trayer, I. P. (1974) *FEBS Lett.* 40, 253-257.
- Syska, H., Wilkinson, J. M., Grand, R. J. A., & Perry, S. V. (1976) *Biochem. J.* 153, 375-387.
- Talbot, J. A., & Hodges, R. S. (1979) *J. Biol. Chem.* 254, 3720-3723.
- Talbot, J. A., & Hodges, R. S. (1981a) *J. Biol. Chem.* 256, 2798-2802.
- Talbot, J. A., & Hodges, R. S. (1981b) *J. Biol. Chem.* 256, 12374-12379.
- Toyo-Oka, T. (1982) *Biochem. Biophys. Res. Commun.* 107, 44-50.
- Van Eerd, J. P., & Takahashi, K. (1975) *Biochem. Biophys. Res. Commun.* 64, 122-127.
- Villar-Palasi, C., & Kumon, A. (1981) *J. Biol. Chem.* 256, 7409-7415.
- Wang, C.-L. A., & Gergely, J. (1986) *Eur. J. Biochem.* 154, 225-228.
- Wilkinson, J. M. (1980) *Eur. J. Biochem.* 103, 179-188.
- Wilkinson, J. M., & Grand, R. J. A. (1975) *Biochem. J.* 149, 493-496.
- Wilkinson, J. M., & Grand, R. J. A. (1978a) *Nature (London)* 271, 31-35.
- Wilkinson, J. M., & Grand, R. J. A. (1978b) *Eur. J. Biochem.* 82, 493-501.
- Yamamoto, K., & Ohtsuki, I. (1982) *J. Biochem. (Tokyo)* 91, 1669-1677.
- Zot, A., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem.* 16, 535-559.

## Trypsin Digestion of Junctional Sarcoplasmic Reticulum Vesicles<sup>†</sup>

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Received October 12, 1987; Revised Manuscript Received December 21, 1987

**ABSTRACT:** A putative constituent of the junctional processes, connecting the terminal cisternae of sarcoplasmic reticulum and the transverse tubules of skeletal muscle fibers, is a  $\geq 350\,000$ -dalton (Da) protein that displays ryanodine binding and  $\text{Ca}^{2+}$  channel properties. Ryanodine modulation of  $\text{Ca}^{2+}$  fluxes suggests that the ryanodine receptor and calcium channel are integral parts of one functional unit corresponding to the  $\geq 350\,000$ -Da protein [Inui, M., Saito, E., & Fleischer, S. (1987) *J. Biol. Chem.* 262, 1740-1747; Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. L., Sutko, J. L., Kahl, S. D., Raab, C. R., & Madson, L. (1987) *J. Biol. Chem.* 262, 6460-6463]. We subjected vesicular fragments of junctional-cisternal membrane to stepwise trypsin digestion. The  $\geq 350\,000$ -Da protein is selectively cleaved in the early stage of digestion, with consequent disappearance of the corresponding band in electrophoretic gels. The  $\text{Ca}^{2+}$ -ATPase is cleaved at a later stage, while calsequestrin is not digested under the same experimental conditions. While the  $\text{Ca}^{2+}$ -ATPase yields two complementary fragments that are relatively resistant to further digestion, the  $\geq 350\,000$ -Da protein yields fragments that are rapidly broken down to small peptides. Under conditions producing extensive digestion of the  $\geq 350\,000$ -Da protein, the junctional processes are still visualized by electron microscopy, with no discernible alterations of their ultrastructure. The functional properties of the  $\text{Ca}^{2+}$  release channel are also maintained following trypsin digestion, including blockage by  $\text{Mg}^{2+}$  and ruthenium red and activation by  $\text{Ca}^{2+}$  and nucleotides. Prolonged incubations with and after trypsin produce functional alterations. Ryanodine, at relatively high (micromolar) concentrations, partitions into a nonsaturable compartment of the membrane and inhibits  $\text{Ca}^{2+}$  efflux through the release channel. On the other hand, high-affinity ryanodine binding ( $K_d = 3 \times 10^{-7}$  M) occurs with a stoichiometry approximating that of the  $\geq 350\,000$ -Da protein and produces a reduction of net  $\text{Ca}^{2+}$  uptake by the vesicles, due to increased  $\text{Ca}^{2+}$  efflux through the release channel. The bound ryanodine does not interfere with digestion of the  $\geq 350\,000$ -Da protein and is still bound following digestion. We suggest that the  $\geq 350\,000$ -Da protein permits entrance of trypsin into a large crevice (likely at the opening of the channel) where multiple cleavage sites are readily available. The resulting proteolytic fragments remain stabilized by multiple noncovalent interactions and are only dissociated by strong detergents. Additional protein components may contribute to structural stabilization of the junctional processes. Ryanodine binds to a protein domain where it does not interfere with trypsin binding but can regulate the channel through allosteric mechanisms.

**C**larification of the mechanism of  $\text{Ca}^{2+}$  release from sarcoplasmic reticulum (SR) is a missing link for a molecular understanding of excitation-contraction coupling in muscle.

<sup>†</sup> This study was supported by grants from the National Institutes of Health (HL-27867) and the Muscular Dystrophy Association (to G.I.) and by an American Heart Association fellowship, California Affiliate (to A.C.). Parts of this study were carried out in the Biology Department of San Diego State University, San Diego, CA, and in the Physiology Department of the University of the Pacific, San Francisco, CA.

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Recent progress has been made in this regard by a series of studies demonstrating that high-density SR vesicles ("heavy" SR) are able to release  $\text{Ca}^{2+}$  rapidly, through a  $\text{Ca}^{2+}$ - and nucleotide-dependent channel which is absent in light SR (Nagasaki & Kasai, 1983; Meissner, 1984; Ikemoto et al., 1985; Chu et al., 1986). Furthermore, membrane material from heavy SR can be incorporated into model bilayers, forming a high-conductance channel which is modulated by  $\text{Ca}^{2+}$  and nucleotides (Smith et al., 1985, 1986; Rousseau et al., 1986). The heavy SR has been identified with vesicular