Extensive Interactions between Troponins C and I. Zero-Length Cross-Linking of Troponin I and Acetylated Troponin C†

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ABSTRACT: Interactions between troponin C (TnC) and troponin I (TnI) play an important role in the Ca^{2+}-dependent regulation of vertebrate striated muscle contraction. Earlier studies have led to the proposal that the "inhibitory region" (residues 96−116) of TnI binds to an α-helical segment of TnC comprising residues 89−100 in the nonregulatory, C-terminal domain. Subsequently, on the basis of the results of zero-length cross-linking, we suggested that the inhibitory region of TnI also interacts with the N-terminal, regulatory domain of TnC [Leszyk, J., Grabarek, Z., Gergely, J., & Collins, J. H. (1990) *Biochemistry 29,* 299−304]. In the present study, we acetylated the ϵ-NH_2 groups of the nine lysines of TnC in order to avoid complications which may arise from intramolecular cross-linking between NH_2 and COOH groups of TnC. We then activated the COOH groups of acetylated TnC (AcTnC) with 1-ethyl-3-[3−(dimethylamino)propyl]carbodiimide and *N*-hydroxysuccinimide. The activated AcTnC was combined with TnI, and zero-length cross-links were formed between COOH groups in AcTnC and lysine ϵ-NH_2 groups in TnI. The cross-linked heterodimer (AcCxI) was cleaved with CNBr and proteases, and the resulting cross-linked peptides were separated by HPLC and then sequenced. Our results show extensive cross-linking between AcTnC and TnI, involving both the N-terminal and C-terminal domains of TnC, as well as the N-terminal, C-terminal, and inhibitory regions of TnI.

Vertebrate striated muscle contraction is regulated by Ca^{2+} and requires the proteins troponin and tropomyosin, located on the actin-containing thin filaments. Troponin is composed of three subunits: troponin C (TnC),[1] which binds Ca^{2+}; TnI, which inhibits actomyosin ATPase activity, and TnT, which binds tropomyosin. Ca^{2+}-dependent changes in the interactions between TnC and TnI are the key events in the activation of contraction. It is thought that Ca^{2+}-dependent structural changes in TnC weaken the interaction of TnI with actin, so that actin activates myosin ATPase and muscle contracts. [for reviews, see Leavis and Gergely (1984), Zot and Potter (1987), Grabarek *et al.* (1992), and Chalovich (1992)]. The amino acid sequences of rabbit fast skeletal muscle TnC (Collins *et al.*, 1973, 1977; Zot *et al.*, 1987a) and TnI (Wilkinson & Grand, 1975; Sheng *et al.*, 1992) are known, and these proteins have served as models for extensive structure−function studies carried out in several laboratories.

While little is known about the three-dimensional structure of TnI, a detailed crystal structure of TnC has been available for some time (Herzberg & James, 1985; Sundaralingam *et al.*, 1985). The four homologous Ca^{2+}-binding sites originally predicted from the amino acid sequence (Collins *et al.*, 1973) are paired within two structurally independent globular domains which are connected by a long, central helix. The N-terminal domain contains two low-affinity sites which bind Ca^{2+} specifically and are responsible for the regulation of muscle contraction. The C-terminal domain contains two high-affinity structural sites which bind either Ca^{2+} or Mg^{2+} and play a structural role (Potter & Gergely, 1975; Leavis *et al.*, 1978; Johnson *et al.*, 1979; Robertson *et al.*, 1981; Putkey *et al.*, 1989). Only the C-terminal sites are occupied in the crystal structure of TnC. A model of Ca^{2+}-induced conformational changes in TnC (Herzberg *et al.*, 1986) postulates that binding of Ca^{2+} to the N-terminal domain of TnC is accompanied by a shift in the relative positions of its helices, resulting in a structure similar to that of the C-terminal domain. This would create a hydrophobic "patch" on the surface of TnC which may bind to TnI. Site-directed mutagenesis of TnC (Grabarek *et al.*, 1990; Fujimori *et al.*, 1990; Wang *et al.*, 1990; Gusev *et al.*, 1991; daSilva *et al.*, 1993) and NMR studies on the N-terminal domain of TnC (Gagne *et al.*, 1994) support this hypothesis. Recent small-angle X-ray and neutron scattering studies (Olah *et*

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[1] Abbreviations: CNBr, cyanogen bromide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NHS, *N*-hydroxysuccinimide; TnC, troponin C; TnI, troponin I; TnT, troponin T; AcTnC, acetylated TnC; AcCxI, cross-linked complex of AcTnC and TnI; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NMR, nuclear magnetic resonance.

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al., 1994), as well as energy transfer and fluorescence quenching data (Gong *et al.*, 1994), indicate that TnC preserves its extended conformation when complexed to TnI.

A number of biochemical investigations of troponin have dealt with the TnC−TnI interface. The equilibrium binding constants for the formation of the TnC−TnI complex are approximately 10^6 and 10^9 M^{-1} in the absence and presence of Ca^{2+}, respectively, although values reported by various authors differ significantly (Ingraham & Swenson, 1984; Leavis *et al.*, 1984; Wang & Cheung, 1985; Cheung *et al.*, 1987). Studies on proteolytic fragments identified three helical regions of TnC that form part of the binding interface with TnI (Grabarek *et al.*, 1981). These are TnC residues 50−60, 89−100, and 126−136, which form the main parts of helices designated C, E, and G, respectively. Studies on cyanogen bromide fragments of TnI have shown that the N-terminal peptide CN5[2] (residues 1−21) and the "inhibitory" peptide CN4 (residues 96−116) bind to whole TnC (Syska & Grand, 1976; Grand *et al.*, 1982). NMR data (Dalgarno *et al.*, 1982) and photo-cross-linking studies (Leszyk *et al.*, 1987, 1988), both using probes attached to Cys-98 of TnC, suggested that the CN4 region of TnI interacts with TnC segment 89−100, which is adjacent to Ca^{2+}-binding site III in the C-terminal, nonregulatory domain. The importance of this interaction in regulation is supported by the observations that (a) proteolytic fragments of TnC that contain residues 89−100 can substitute for intact TnC in Ca^{2+}-sensitization of the reconstituted actomyosin complex (Weeks & Perry, 1978; Grabarek *et al.*, 1981) and (b) peptide CN4 of TnI mimics intact TnI in its ability to inhibit actomyosin ATPase (Syska *et al.*, 1976; Talbot & Hodges, 1981; Nozaki *et al.*, 1980). On the other hand, TnC mutants with some deletions in segment 89−100 retain their regulatory properties (Dobrowolski *et al.*, 1991).

Previously, we (Leszyk *et al.*, 1990) found that the main product of zero-length cross-linking between TnC and TnI comprises segments derived from the N-terminal, regulatory domain of TnC (residues 46−78) and the inhibitory region of TnI (residues 96−116). The absence of cross-links between TnI and residues 89−100 of TnC could be due to participation of activated TnC β- and γ-COOH groups in forming intramolecular cross-links prior to complex formation with TnI. Such internal cross-linking of TnC upon incubation with EDC has been demonstrated (Grabarek *et al.*, 1989); γ-COOH groups of Glu-92, -93 and -94 are likely to be involved due to their proximity to ϵ-NH_2 groups of Lys-88 and -90. In the present study, we eliminated the possibility of internal cross-linking by acetylating the nine Lys residues of TnC prior to reaction with EDC and cross-linking with TnI. Characterization of the product, AcCxI, revealed extensive cross-linking between TnC and TnI, involving both the N-terminal and C-terminal domains of TnC, as well as the N-terminal, C-terminal, and inhibitory regions of TnI.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest grade commercially available. Sequencer reagents were from Applied Biosystems. PTH-amino acid standards were from either Pierce or Applied Biosystems. Amino acid standards, PITC (for nonsequencer use), constant-boiling HCl, and CNBr were obtained from Pierce. TPCK-treated trypsin was obtained from Worthington. *Staphylococcus aureus* V8 protease was from ICN Immuno Biologicals. Lysyl endopeptidase was from Wako Chemicals. HPLC-grade H_2O and TFA were obtained from Fisher. HPLC-grade acetonitrile was obtained from Burdick and Jackson. All other chemicals were obtained from Fisher.

Acetylation of TnC. Rabbit skeletal TnC and TnI were prepared by the method of Greaser and Gergely (1971). TnC was acetylated with acetic anhydride essentially according to Fraenkel-Conrat (1957). Six 1 mL aliquots of acetic anhydride were added at 10 min intervals to the protein dissolved in 50%-saturated sodium acetate (2 mg/mL) at 0 °C. Then the samples were dialyzed overnight *vs* 1 mM $NaHCO_3$. *O*-Acetyltyrosine formed was deacetylated by incubation with hydroxylamine (Riordan & Vallee, 1972).

Preparation of Cross-Linked Protein. TnI and AcTnC were cross-linked using the two-step, zero-length cross-linking procedure of Grabarek and Gergely (1990), as described previously (Leszyk *et al.*, 1990). The presence of the covalently cross-linked complex AcCxI was verified by electrophoresis on a 10% polyacrylamide gel (not shown) in the presence of 5 M urea and 1 mM EDTA. The absence of bands corresponding to free TnC and TnI indicated a 100% yield of cross-linking. The new band was rather diffuse, possibly owing to heterogeneity of the cross-linked material with respect to the number and location of cross-linking sites (Leszyk *et al.*, 1990).

Preparation of Cross-Linked Peptides. S-Pyridylethylation of protein thiol groups (Fullmer, 1984) in AcCxI, cleavage into peptides with CNBr, lysyl endopeptidase and *S. aureus* V8 protease, and separation of peptides by reverse-phase HPLC were carried out as described previously (Kobayashi *et al.*, 1994).

Amino Acid Compositions and Sequence Analysis. Amino acid compositions were determined by the Waters "PICO-Tag" method, as described previously (Collins *et al.*, 1988). Amino acid sequences were determined with an Applied Biosystems Model 477A Protein Sequencer, equipped with an on-line Model 120A PTH amino acid analyzer.

RESULTS

Digestion of AcCxI with CNBr. HPLC of a CNBr digest of AcCxI yielded, as in our previous cross-linking studies (Leszyk *et al.*, 1987, 1988, 1990), a broad, late-eluting peak of cross-linked peptides (Figure 1). Sequence analysis of this fraction gave complex results, but we could identify three peptides from TnC (CB7−CB9) and five from TnI (CN1−CN4, CN7) (see Table 1, Figure 6, and Figure 7). Yields for some of the peptides could not be quantified because of their low abundance and the complexity of the results. TnC peptide CB8 (residues 46−78), which is usually insoluble at the low pH values we use for HPLC (Collins *et al.*, 1977; Collins, 1980), was found in the highest yield in this fraction. This indicates that CB8 was solubilized by cross-linking to basic peptide(s) from TnI, as we had observed previously (Leszyk *et al.*, 1990). TnC peptide CB9 (residues 84−135) was shown previously to coelute with the cross-linked fraction (Leszyk *et al.*, 1990); therefore, additional evidence

[2] In keeping with the usual practice, CNBr peptides from TnC and TnI are referred to by the names given in the initial reports of their amino acid sequences. CNBr peptides from TnC are designated CB (Collins *et al.*, 1977), while those from TnI are designated CN (Wilkinson & Grand, 1975).

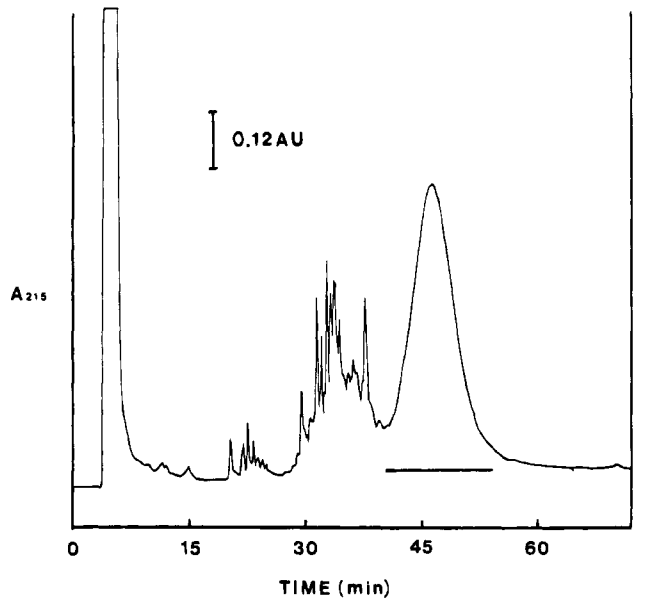

FIGURE 1: HPLC elution profile of a CNBr digest of AcCxI, using a Vydac 214TP54 (C_4) column. The late-eluting, broad fraction indicated by the horizontal bar contained cross-linked material and was subjected to sequence analysis.

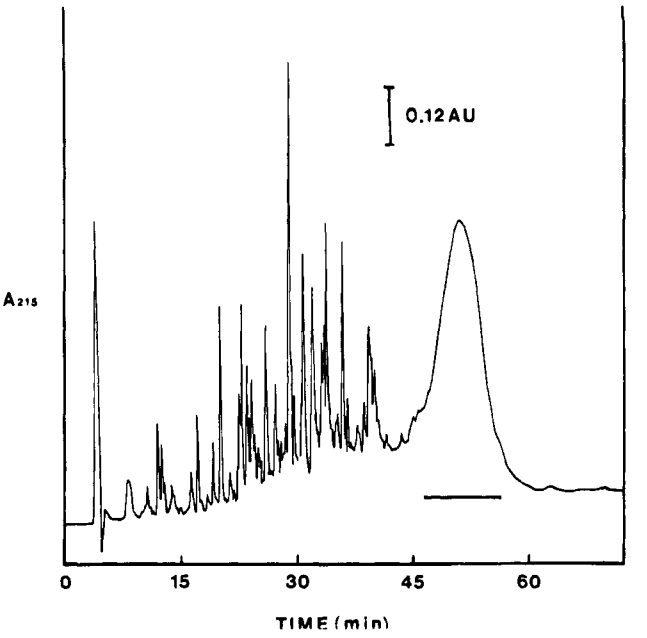

FIGURE 2: HPLC elution profile of a *S. aureus* V8 protease digest of AcCxI, using a Vydac 218TP54 (C_{18}) column. The late-eluting, broad fraction indicated by the horizontal bar contained cross-linked material and was subjected to sequence analysis.

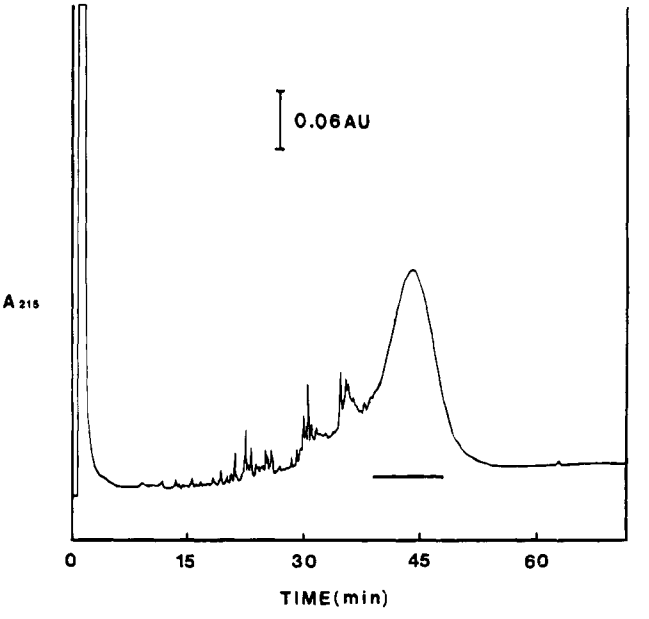

FIGURE 3: HPLC elution profile of a CNBr digest of the V8 protease cross-linked fraction shown in Figure 2, using a Vydac 218TP54 (C_{18}) column. The late-eluting, broad fraction indicated by the horizontal bar contained cross-linked material and was subjected to sequence analysis.

was obtained in the present study to verify cross-linking in this region (see below).

The most abundant TnI sequence found in the cross-linked CNBr fraction of AcCxI was that of CN7 (residues 122–134). This contrasts with our previous zero-length cross-linking study on nonacetylated TnC and TnI, in which CN7 was found in very low yield and the inhibitory peptide CN4 (residues 96–116) was the major component of the cross-linked fraction (Leszyk *et al.*, 1990). In the present study, we also found cross-linked CNBr peptides from the N-terminal region of TnI (CN1 and CN3) which had not been identified previously (Leszyk *et al.*, 1987, 1988, 1990; Kobayashi *et al.*, 1991, 1994). Cross-linking of CN1 to TnC is consistent with studies showing that peptides of various lengths derived from the N-terminal 40 residues of TnI interact with TnC (Syska *et al.*, 1976; Katayama & Nozaki, 1982; Sheng *et al.*, 1992; Ngai & Hodges, 1992; Farah *et al.*, 1994). Cross-linking between the CN3 region of TnI (residues 58–81) and TnC is consistent with the decrease in reactivity of Lys-70 of TnI upon interaction with TnC (Hitchcock-DeGregori, 1982).

Digestion of AcCxI with S. aureus V8 protease. HPLC of a *S. aureus* V8 protease digest of AcCxI once again yielded the characteristic broad, late-eluting cross-linked peptide fraction (Figure 2). Sequence analysis of this fraction (summarized in Figures 6 and 7) identified four TnC peptides (beginning at Leu-39, Leu-55, Phe-75, and Cys-98), derived from both the N-terminal and C-terminal domains, and three TnI peptides (beginning at Leu-63, Ile-83, and Leu-92). Further cleavage with CNBr, and sequence analysis of the resulting cross-linked fraction (Figure 3), yielded sequences corresponding to all of the peptides obtained after CNBr digestion of AcCxI, except for CB7 and CN1. This result confirms cross-linking in the regions corresponding to TnI peptides CN3, CN4, and CN7 and the CB8 region of TnC. Most significantly, the presence of sequences originating from peptide CB9 of TnC (residues 85–135) in the cross-linked fractions obtained after digestion with V8 protease indicates that one or several COOH groups in this region are indeed cross-linked to TnI. This conclusion is based on the fact that, owing to its high content of Glu, the un-cross-linked CB9 region is digested by V8 protease into a number of short peptides which, if not cross-linked to TnI, would be eluted from the HPLC column much earlier than the cross-linked fraction (Leszyk *et al.*, 1990). The lack of TnI sequences from the N-terminal third of TnI in the V8 protease cross-linked fraction may be caused by the complexity of the sequence analysis and/or the low abundance of potential cross-linking sites (only 4 Lys residues among the first 64 amino acids) in this region.

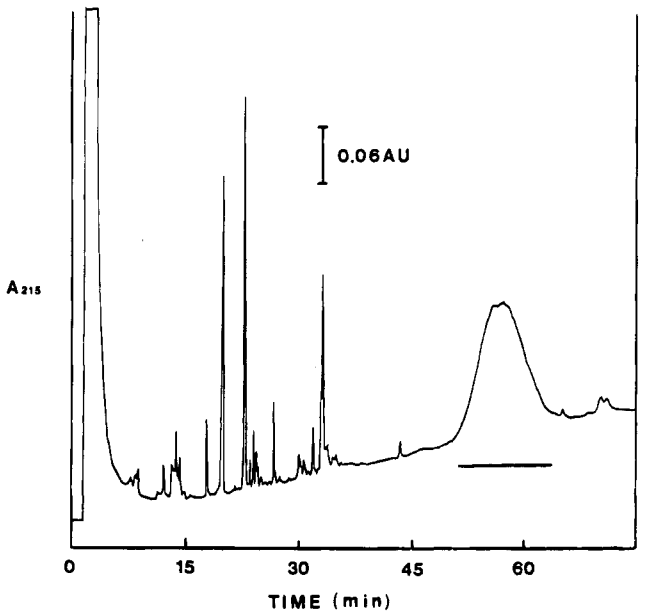

FIGURE 4: HPLC elution profile of a lysyl endopeptidase digest of AcCxI, using a Vydac 218TP54 (C_{18}) column. The late-eluting, broad fraction indicated by the horizontal bar contained cross-linked material and was subjected to sequence analysis.

Table 1: Relative Molar Yields of Peptide Sequences Obtained from the Cross-Linked Fraction of the CNBr Digest of AcCxI

	peptide	residues	AcCxI	CxI[a]
TnC	CB8	46−78	1.0[b]	1.0[b]
	CB9	84−135	0.7	0.8[c]
	CB7	136−154	NQ[d]	
TnI	CN1	22−57	NQ	
	CN3	58−81	0.4	
	CN4	96−116	0.4	0.5
	CN7	122−134	0.7	0.2
	CN2	135−170	NQ	0.2

[a] data from Leszyk *et al.* (1990). [b] Yields are normalized with respect to CB8 peptide. [c] Not cross-linked, but coeluted with cross-linked fraction (Leszyk *et al.*, 1990). [d] NQ, not quantified; yields less than 0.4 mol/mol of CB8.

Digestion of AcCxI with Lysyl Endopeptidase. We also digested AcCxI with lysyl endopeptidase. The only sites available for cleavage with this protease were the un-cross-linked Lys residues in TnI, since all the ϵ-NH_2 groups of AcTnC were blocked by acetylation. The HPLC profile of this digest (Figure 4) shows, in addition to the usual broad, late-eluting peak of cross-linked material, several sharp peaks which were all identified by amino acid analysis as TnI peptides. Sequence analysis of the cross-linked fraction, which presumably consists of intact TnC and covalently attached peptides from TnI, also yielded only TnI sequences. This was expected, since the N-terminus of TnC is blocked (Collins *et al.*, 1973, 1977). A total of eight cross-linked TnI peptides could be recognized (see Figure 7), including one (beginning at Arg-6) from the N-terminus that was not detected in the cross-linked fraction from the V8 protease digest. The other seven cross-linked lysyl endopeptidase peptides overlapped with the five CNBr peptides of TnI listed in Table 1. Three of these (beginning at Glu-91, Leu-99, and Phe-106) overlapped CN4 (see Figure 7), the inhibitory region of TnI, and the sum of their yields exceeded the yields of peptides from other regions of TnI. We further cleaved the cross-linked fraction from the lysyl endopeptidase digest with CNBr (Figure 5). TnC peptides CB8 and CB9, as well as TnI peptides starting at Arg-6, Leu-99, and Ala-124, were identified by sequence analysis of the resulting cross-linked fraction (Figure 7). Several other peptides appeared to be present, but these could not be identified because of their low yields.

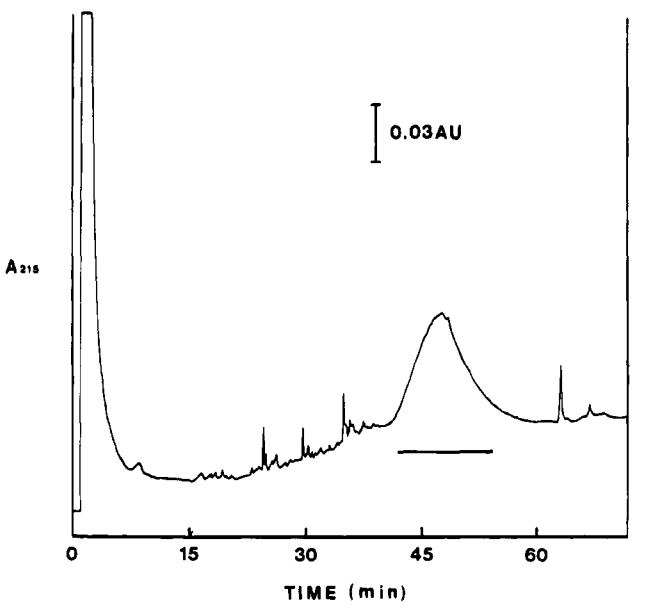

FIGURE 5: HPLC elution profile of a CNBr digest of the lysyl endopeptidase cross-linked fraction shown in Figure 4, using a Vydac 218TP54 (C_{18}) column. The late-eluting, broad fraction indicated by the horizontal bar contained cross-linked material and was subjected to sequence analysis.

DISCUSSION

In this and a previous (Leszyk *et al.*, 1990) study, we used a two-step procedure (Grabarek & Gergely, 1990) in which EDC induces formation of NHS esters (so-called active esters) with the COOH groups of Asp and Glu side chains of TnC or AcTnC. After inactivation of the excess EDC with β-mercaptoethanol, an equimolar amount of TnI was added to form a complex with TnC. This led to the formation of isopeptide bonds between Lys NH_2 groups in TnI and nearby activated COOH groups in TnC. An advantage of the two-step procedure is that only one component of the complex is exposed to EDC, thus uniquely defining the origin of COOH groups in the cross-links. The use of AcTnC in the present study eliminates intramolecular cross-linking within TnC, which could interfere with cross-linking to TnI.

The results of sequence analyses of the various cross-linked fractions are summarized in Figures 6 and 7. The results from each fraction were complex, revealing as many as eight simultaneous sequences. This prevented us from identifying individual cross-linked residues, although we could identify several specific regions of cross-linking.

In AcTnC, the most abundant sequences in the cross-linked fractions were those corresponding to Ca^{2+}-binding sites II (CB8) and III (CB9). The latter, while not detected in our previous study (Leszyk *et al.*, 1990), was recovered here in good yield from all digests, clearly indicating that it was cross-linked to TnI. Peptide CB7 (Ca^{2+}-binding site IV) was also detected in the CNBr digest, although its yield was low and we could not detect this sequence in the V8 protease digest or in the lysyl endopeptidase + CNBr digest. Thus,

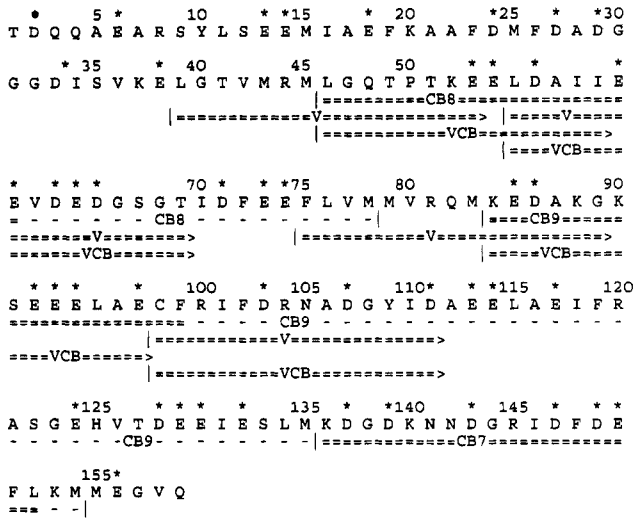

FIGURE 6: Amino acid sequence of rabbit fast skeletal muscle TnC (Collins *et al.*, 1977; Zot *et al.*, 1987), and summary of TnC sequences identified in cross-linked fractions obtained after digestion of AcCxI with CNBr [designated CB, in accordance with the original nomenclature of Collins *et al.* (1977)], V8 protease (V), and V8 protease followed by CNBr (VCB). Double underlines indicate residues identified by sequence analysis. The location of each Asp (D) or Glu (E) residue is highlighted by an asterisk.

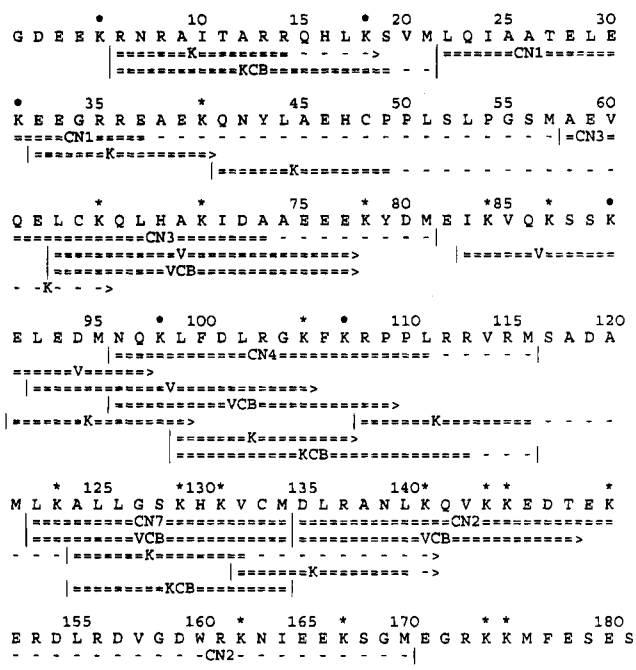

FIGURE 7: Amino acid sequence of rabbit fast skeletal TnI, and summary of TnI sequences identified in cross-linked fractions obtained after digestion of AcCxI with CNBr [designated CN, in accordance with the original nomenclature of Wilkinson and Grand (1975)], lysyl endopeptidase (K), lysyl endopeptidase followed by CNBr (KCB), V8 protease (V), and V8 protease followed by CNBr (VCB). Double underlines indicate residues identified by sequence analysis. The location of each Lys (K) residue is highlighted by an asterisk. Note: The sequence shown includes the recently identified (Sheng *et al.*, 1992) tripeptide Leu-Arg-Asp (L-R-D), residues 155–157, which was overlooked in the original, 178-residue sequence of Wilkinson and Grand (1975).

it seems likely that cross-linking in this region was nonspecific. Residues in CB8 have been shown previously to cross-link to TnI using zero-length (Leszyk *et al.*, 1990) and benzophenone (Kobayashi *et al.*, 1991) cross-linking methods. Residues in the CB9 segment have been cross-linked to TnI previously *via* formation of a disulfide bond (Dobrovol'sky *et al.*, 1984) or with a benzophenone photo-cross-linker (Leszyk *et al.*, 1987; Kobayashi *et al.*, 1994).

The cross-linked segments of TnI span most of the molecule with the exception of the C-terminal 36 residues (146–181), which showed no evidence of cross-linking. The most abundant TnI sequences in the cross-linked fractions were those derived from the highly positively charged 50-residue segment (residues 96–145) comprising CNBr peptides CN4 (the inhibitory segment), CN7, and a part of CN2. Cross-linking of this region of TnI to TnC by various methods including disulfide formation (Dobrovol'sky *et al.*, 1984), zero-length (Leszyk *et al.*, 1990), and photo-cross-linking (Leszyk *et al.*, 1987; Kobayashi *et al.*, 1991, 1994) has been reported. In the present work, we found additional TnI cross-linked sites in the N-terminal 40 residues and in the region corresponding to peptide CN3 (residues 58–81). Although interactions between TnC and TnI peptides derived from the N-terminal 40 residues have been reported previously (Syska *et al.*, 1976; Grand *et al.*, 1982; Dalgarno *et al.*, 1982; Katayama & Nozaki, 1982; Ngai & Hodges, 1992), cross-linking evidence was not available. Cross-linking of peptide CN3 to TnC is consistent with the finding that the accessibility of TnI Lys-70 to acetylation is significantly decreased upon interaction of TnI with TnC (Hitchcock-DeGregori, 1982).

Our results clearly show more extensive intermolecular cross-linking in the AcTnC–TnI complex than in the TnC–TnI complex. There seem to be two plausible explanations for this. It is possible that, in TnC–TnI, some cross-links may not have been formed because of interference from intramolecular cross-linking in TnC as previously suggested (Leszyk *et al.*, 1990). Another possibility is that acetylation of TnC creates contact sites that do not exist in the native complex. Intramolecular zero-length cross-linking in TnC has been documented (Grabarek *et al.*, 1989), which would favor the first possibility. On the other hand, several properties of TnC are altered upon acetylation (Grabarek *et al.*, 1995). AcTnC has a lowered stability in the absence of Ca^{2+}, an altered affinity for Ca^{2+}, and an increased affinity for TnT, TnI, and myofibrils. None of these changes, however, imply the formation of new interaction sites. Moreover, the increased affinity for TnI appears to be confined to the N-terminal domain of AcTnC (Grabarek *et al.*, 1995), in which we did not detect any effects of acetylation on zero-length cross-linking. Therefore, we believe that the cross-linked sites in the AcTnC–TnI complex identified in the present work are representative of the native complex. Even if minor differences in the interaction exist, the general conclusions reached for AcTnC are likely to be valid for the native complex since AcTnC is fully capable of substituting for native TnC in the Ca^{2+}-dependent regulation of myofibrillar ATPase (Grabarek *et al.*, 1995).

Our finding of extensive cross-linking between TnC and TnI is consistent with a recent model of the TnC–TnI binary complex based on small-angle X-ray and neutron scattering data (Olah *et al.*, 1994; Olah & Trewhella, 1994). This model proposes that TnI contains three structural regions: a central (residues ~50– 130), largely α-helical region which winds in a spiral around the full length of TnC, and two end regions which bind to the outer edges of the N- and C-terminal domains of TnC. In this model, TnC retains the

extended conformation seen in its crystal structure, with two domains separated by a long, central helix. Our data are also consistent with a model of the TnC−TnI complex proposed by Farah *et al.* (1994), in particular with their suggestion that a large stretch of residues C-terminal to the inhibitory segment of TnI is also involved in the interaction with TnC. Perhaps the most significant result of our present and previous cross-linking studies is that the inhibitory segment of TnI (residues 96−116) is in contact with both domains of TnC. Whereas studies on various fragments of TnI and TnC have suggested this possibility (Tsuda *et al.*, 1991; Swenson & Fredricksen, 1992; Farah *et al.*, 1994), only our cross-linking studies have shown this to be the case for the full-length proteins. In our previous cross-linking studies (Leszyk *et al.*, 1987; Kobayashi *et al.*, 1991, 1994), we found that the photoactivatable reagent benzophenone-4-maleimide attached to Cys residues located at positions 12, 57, 89, and 98 of TnC cross-linked with TnI segments 132−141, 113−121, 108−113, and 103−111, respectively. These data provided evidence for an antiparallel orientation of TnI segment 103−141 with respect to TnC in the TnC−TnI complex. Such an antiparallel arrangement apparently extends to the whole complex, as was recently concluded on the basis of studies using recombinant TnI and its fragments (Sheng *et al.*, 1992; Farah *et al.*, 1994; Krudy *et al.*, 1994).

Finally, we would like to point out that zero-length cross-linking detects only ionic interactions between COO^- and NH_3^+ groups, and is unable to detect hydrophobic interactions or other types of polar interactions. Thus, identification of zero-length cross-linking sites is not equivalent to delineation of the interaction interfaces. It is clear that the key Ca^{2+}-induced regulatory event in TnC is the opening of the hydrophobic pocket in the N-terminal domain as proposed in the Herzberg−Moult−James model (Herzberg *et al.*, 1986). Thus, the interacting surface on TnI must also be mostly hydrophobic. This is indeed the case in the complex of calmodulin (CaM), a protein very similar to TnC, with a synthetic peptide, M13, based on the CaM-binding site of myosin light chain kinase (Ikura *et al.*, 1992; Meador *et al.*, 1992). In this complex, the positively charged, amphipathic helical peptide M13 is located in a hydrophobic channel formed by the two domains of CaM. The peptide is anchored into CaM mostly by bulky hydrophobic side chains of Trp and Leu, but there are also ionic interactions between Glu residues of CaM and Lys residues of the peptide at the outer rim of both domains of CaM. It is likely that at the TnC−TnI interface there is a similar combination of hydrophobic and polar interactions in spite of a presumably different (extended rather than compact) overall structure. It is clear that a high-resolution crystal structure of the troponin complex is needed to further our understanding of the mechanism of regulation of contraction.

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