Characterization of Zero-Length Cross-Links between Rabbit Skeletal Muscle Troponin C and Troponin I: Evidence for Direct Interaction between the Inhibitory Region of Troponin I and the NH_2-Terminal, Regulatory Domain of 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. Previous attempts to elucidate the molecular details of TnC–TnI interactions, mainly involving chemically modified proteins or fragments thereof, have led to the widely accepted idea that the "inhibitory region" (residues 96–116) of TnI binds to an α-helical segment of TnC comprising residues 89–100 in the nonregulatory, COOH-terminal domain. In an attempt to identify other possible physiologically important interactions between these proteins, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was used to produce zero-length cross-links in the complex of rabbit skeletal muscle TnC and TnI. TnC was activated with EDC and *N*-hydroxysuccinimide (NHS) and then mixed with an equimolar amount of TnI [Grabarek, Z., & Gergely, J. (1988) *Biophys. J. 53*, 392a]. The resulting cross-linked TnC×I was cleaved with cyanogen bromide, trypsin, and *Staphylococcus aureus* V8 protease (SAP). Cross-linked peptides were purified by reverse-phase HPLC and characterized by sequence analysis. The results indicated that residues from the regulatory Ca^{2+}-binding site II in the NH_2-terminal domain of TnC (residues 46–78) formed cross-links with TnI segments spanning residues 92–167. The most highly cross-linked residues in TnI were Lys-105 and Lys-107, located in the inhibitory region. These results yield the first evidence for an interaction between the N-terminal domain of TnC and the inhibitory region of TnI.

The Ca^{2+}-dependent change in interaction between the Ca^{2+}-binding (TnC)[1] and the inhibitory (TnI) components of troponin is one of the key events in the process of activation of contraction in skeletal muscle [for reviews, see Leavis and Gergely (1984) and Zot and Potter (1987)]. In order to understand the molecular basis of muscular activation, much effort has been made to characterize the TnC–TnI interface. The amino acid sequences of rabbit fast skeletal muscle TnC (Collins et al., 1973, 1977) and TnI (Wilkinson & Grand, 1975) are known, and these proteins have served as models for extensive structure–function studies carried out in several laboratories. While much remains to be learned about the three-dimensional structure of TnI, 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 later confirmed by the crystal structures of chicken (Sundaralingam et al., 1985) and turkey (Herzberg & James, 1985) TnCs, which also revealed the presence of a long, central helix linking structurally independent domains comprising each half of the molecule.

Evidence collected to date points to multiple interactions between TnC and TnI, involving segments located at some distance from each other in the primary structures (Syska et al., 1976; Grabarek et al., 1981). Of particular functional importance appears to be the interaction between the inhibitory segment of TnI (residues 96–116) and an α-helical segment of TnC (residues 89–100) adjacent to Ca^{2+}-binding site III. A CNBr fragment (CN4) of TnI that comprises residues 96–116 mimics intact TnI in its ability to inhibit actomyosin ATPase (Syska et al., 1976; Talbot & Hodges, 1981; Nozaki et al., 1980), whereas the presence of residues 89–100 in proteolytic fragments of TnC has been found to be essential for their ability to substitute for intact TnC in Ca^{2+} sensitization of the reconstituted actomyosin complex (Weeks & Perry, 1978; Grabarek et al., 1981). The most convincing evidence for the interaction between the two segments has been provided by ^1H NMR data (Dalgarno et al., 1982) and by

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NHS, *N*-hydroxysuccinimide; TnC, troponin C; TnI, troponin I; TnT, troponin T; TnC×I, cross-linked complex of TnC and TnI; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SAP, *Staphylococcus aureus* V8 protease; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TPCK, *N*-tosylphenylalanine chloromethyl ketone; NMR, nuclear magnetic resonance.

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photo-cross-linking (Leszyk et al., 1987, 1988) utilizing modification of TnC Cys-98 with a spin probe and a benzophenone photo-cross-linker, respectively. Little is known about other sites implicated in the TnC–TnI interaction and about their functional importance.

In the present study, we have utilized zero-length cross-linking with EDC to further characterize the TnC–TnI interface. The lack of specificity of EDC and the requirement for direct contact between COOH groups in TnC and lysine NH_2 groups in TnI, presumably present at the interface, were expected to provide new information. We found that the main product of cross-linking involves a segment of TnC corresponding to the CNBr fragment CB8 (residues 46–78) and the inhibitory segment of TnI (residues 96–116). Some lysines C-terminal to residue 116 in TnI were also cross-linked to CB8. Rather surprisingly, no evidence was found for cross-linking between TnI and the 89–100 segment of TnC. The results are discussed in light of current views about the three-dimensional structure of TnC based on X-ray crystallographic and solution physicochemical data.

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. SAP was from ICN Immuno Biologicals. HPLC-grade water and TFA were obtained from Fisher. HPLC-grade acetonitrile was obtained from Burdick and Jackson. All other chemicals were obtained from Fisher.

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 gas-phase protein sequencer, equipped with an on-line Model 120A PTH amino acid analyzer.

Preparative HPLC. All peptide mixtures were separated by reverse-phase HPLC using either a 4.6 mm × 25 cm Vydac 218TP54 (C_{18}) or a 214TP54 (C_4) column. Two Waters M510 pumps, a Waters M680 controller, a Waters M480 variable-wavelength absorbance detector, a Linear dual-channel recorder, and a Glenco SV-3 injector were used as our HPLC 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). All separations were carried out by using a linear gradient from 0% B to 50% B over 60 min at a flow rate of 1 mL/min. The eluent was monitored at 220 nm.

Preparation of Cross-Linked Peptides. Rabbit skeletal TnC and TnI were prepared by the method of Greaser and Gergely (1971) and cross-linked as described by Grabarek and Gergely (1988). TnC (21.4 mg), in 18 mL of 0.1 M KCl, 20 mM MES, pH 6.0, 0.2 mM $CaCl_2$, and 5 mM *N*-hydroxysuccinimide (NHS), was activated by incubation with 5 mM EDC (final concentration) for 15 min at 25 °C. The activation step was terminated by addition of β-mercaptoethanol (20 mM final concentration), and 25 mg of rabbit skeletal troponin I in 4.6 mL of a solution containing 0.4 M KCl and 0.23 M Hepes, pH 7.5, was added. The protein solution was further incubated for 2 h at 25 °C to induce cross-linking. The presence of a covalently cross-linked complex was verified by electrophoresis on a 10% polyacrylamide gel in the presence of 5 M urea (Tris–glycine buffer, pH 8.6, and 1 mM EDTA). The absence of a band corresponding to free TnC indicated a 100% yield of cross-linking. The protein solution was dialyzed against water, lyophilized, and used for identification of the cross-linking sites.

For S-pyridylethylation of thiol groups (Fullmer, 1984) in the resulting TnC×I, a sample containing 60–70 nmol of protein was dissolved in 300 μL of alkylation buffer (6 M urea, 0.25 M Tris-HCl, and 1 mM EDTA, pH 8.5). To this was added a 100-fold molar excess of β-mercaptoethanol over SH groups. The reduction took place for 1 h under N_2. A 200-fold molar excess of 4-vinylpyridine over thiol groups was then added, and the reaction took place for 1.5 h under N_2. The alkylated protein was then desalted by reverse-phase HPLC using a Vydac 214TP54 column using the system described above with a linear gradient from 0% to 100% B in 30 min.

For CNBr digestion, a 35-nmol sample of pyridylethylated TnC×I was dissolved in 200 μL of 70% formic acid. To this was added 7 mg of CNBr (freshly dissolved in 7 μL of 70% formic acid). Digestion occurred at room temperature with constant stirring for 19 h. The reaction mixture was diluted to 2 mL with water and dried under N_2. The sample was then dissolved in 200 μL of 70% formic acid and applied directly to the HPLC column. The cross-linked fraction (29 nmol) obtained from the CNBr digest was dissolved in 1.6 mL of buffer (20 mM Na_2HPO_4/4 M urea, pH 8.5). To this was added 6 μL of citraconic anhydride over a 30-min period while maintaining the pH between 8 and 9. For desalting, the sample was centrifugally filtered through a YM-10 membrane on an Amicon Centicon-10 microconcentrator using 50 mM NH_4HCO_3, pH 8.0, as the exchange solvent. The retentate, in 300 μL of 50 mM NH_4HCO_3, pH 8.0, was digested with 20 μg of TPCK-trypsin for 4 h at room temperature with constant stirring. The digestion was stopped, and citraconyl groups were removed by addition of 100 μL of glacial acetic acid. The mixture was stirred overnight at room temperature before direct application to HPLC.

For SAP digestion, a 29-nmol sample of pyridylethylated TnC×I was dissolved in 250 μL of digest buffer (0.1 M NH_4HCO_3, 2 mM EDTA, and 4 M urea, pH 7.8). To this was added 30 μg of SAP in 30 μL of 0.2 M NH_4HCO_3/4 mM EDTA, pH 7.8. Digestion occurred for 23 h at 37 °C with constant stirring and was stopped by addition of 93 μL of glacial acetic acid. The sample was then applied directly to HPLC. The cross-linked fraction obtained from the SAP digest was dissolved in 200 μL of 70% formic acid. To this was added 2 mg of CNBr dissolved in 70% formic acid. The reaction occurred at room temperature with constant stirring for 19 h. The reaction mixture was diluted to 2 mL with water and dried under N_2. The sample was then dissolved in 100 μL of 70% formic acid and applied directly to HPLC.

Control samples of un-cross-linked, pyridylethylated TnI and TnC were digested and applied to HPLC under the same conditions used for TnC×I.

Results

Analysis of the TnC×I complex by gel electrophoresis (Figure 1) indicated that cross-linking between TnC and TnI was essentially complete, since there were no significant bands associated with either TnC or TnI. The new band of cross-linked material was rather diffuse, possibly due to heterogeneity of the cross-linked residues.

HPLC elution profiles of CNBr digests of TnC×I were essentially identical with those of an equimolar mixture of TnC and TnI (Figure 2) except for the appearance of a broad, cross-linked fraction which coeluted with the TnC peptide CB9. Amino acid analyses of the un-cross-linked fractions permitted the identification of all the major (six or more

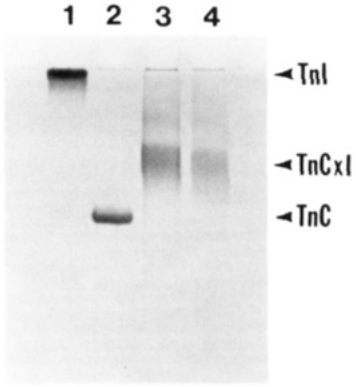

FIGURE 1: Gel electrophoresis of TnI (lane 1), TnC (lane 2), and TnC×I (lanes 3 and 4). A 10% polyacrylamide gel was run in the presence of 5 M urea, 80 mM Tris–glycine buffer, pH 8.6, and 1 mM EDTA.

Table I: Designation of CNBr Peptides of TnC and TnI[a]

TnI	TnC	TnI	TnC
CN5 (1–21)	CB5 (1–15)	CN10 (117–121)	CB3 (80–83)
CN1 (22–57)	CB4 (16–25)	CN7 (122–134)	CB9 (84–135)
CN3 (58–81)	CB6 (26–43)	CN2 (135–167)	CB7 (136–154)
CN6 (82–95)	CB1 (44–45)	CN8 (168–173)	CB2 (156–159)
CN4 (96–116)	CB8 (46–78)	CN9 (174–178)	

[a] CN designates CNBr peptides of TnI (Wilkinson & Grand, 1978), and CB designates CNBr peptides of TnC (Collins et al., 1977).

Table II: Sequence Analyses of Cross-Linked Fractions[a]

digest	peptide	rel yield, X
CNBr (Figure 2)	TnC: CB8 [46–67]	1.00
	TnC: CB9 [84–105]	0.75
	TnI: CN4 [96–116]	0.50
	TnI: CN7 [122–134]	0.20
	TnI: CN2 [135–156]	0.20

digest	peptide	rel yield X1	rel yield X2	rel yield X3
CNBr + Trypsin (Figure 3)	TnC: CB8 [46–78]	1.00	1.00	1.00
	TnI: [104–112]	0.33	0.52	0.50
	TnI: CN7 [122–134]	0.30	0.30	0.33
	TnI: [138–152]	0.30	0.17	0.10

digest	peptide	rel yield, X
SAP (Figure 4)	TnC: [39–63]	1.00
	TnC: [55–74]	0.44
	TnI: [92–116]	0.56

digest	peptide	rel yield, X
SAP + CNBr (Figure 5)	TnC: [46–65]	1.00
	TnC: [55–67]	0.33
	TnI: CN4 [96–115]	0.47
	TnI: CN7 [122–134]	0.33
	TnI: [135–149]	0.13

[a] Numbers in brackets are residues identified by sequence analysis.

residues) CNBr peptides (see Table I) of both proteins except for CB8 of TnC. CB8 is known to be insoluble at acidic pH (Collins et al., 1977; Collins, 1980), and so was not recovered from HPLC under the conditions used here. The only fractions which appeared to be reduced after cross-linking were those containing the TnI peptides CN4, CN7, and possibly CN2.

Sequence analysis of the cross-linked CNBr fraction revealed the presence of several peptides (see Table II): CB8 and CB9 of TnC and the same three TnI peptides (CN4, CN7, and CN2) whose yields appeared to decrease after cross-linking. Since CN4, CN7, and CN2 normally elute much earlier, their presence in the cross-linked fraction shows that they are cross-linked to TnC peptide(s). Yields from the sequencer (Table II) indicated that CN4 was the most highly

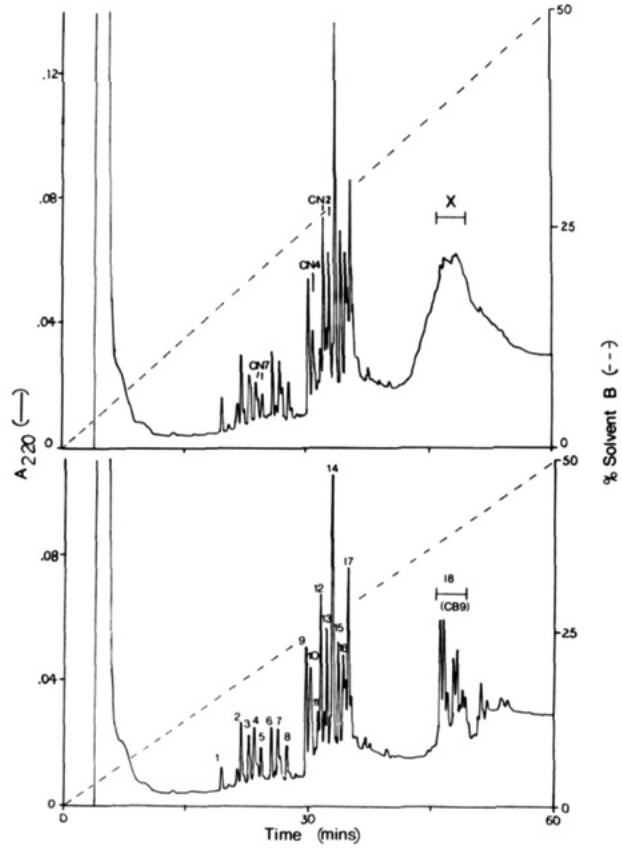

FIGURE 2: HPLC elution profile of CNBr digests of TnC×I (upper panel) and an equimolar mixture of un-cross-linked TnC and TnI (lower panel). Approximately 2 nmol of digested protein was injected onto a Vydac 214TP54 (C_4) column. The identities of the numbered fractions are as follows: 1, CN6; 2, CN5; 3, CN5; 4, CN7; 5, CN7; 6, CB5; 7, CB5; 8, CB5; 9, CB4; 10, CN4; 11, CB6; 12, CN2; 13, CN2; 14, CN3; 15, CN1; 16, CN1; 17, CB7; 18, CB9 (several peaks). CN designates CNBr peptides of TnI (Wilkinson & Grand, 1978), and CB designates CNBr peptides of TnC (Collins et al., 1977). Some peptides eluted as more than one peak, due in part to homoserine to homoserine lactone conversion. The late-eluting broad fraction (designated by an X) in the digest of TnC×I contained cross-linked peptides (see text).

cross-linked TnI peptide. The CB8 sequence was present in the highest amount overall, strongly suggesting that the very acidic CB8 (which was not recovered from un-cross-linked TnC) had been solubilized by cross-linking to basic TnI peptides. The presence of CB9 in the cross-linked fraction was due to coelution of the un-cross-linked peptide (see below).

The cross-linked CNBr fraction was then digested with trypsin. Cleavage was restricted to arginine by reversibly blocking lysine side chains with citraconic anhydride. HPLC of this digest yielded several sharp peaks and another broad, late-eluting fraction (Figure 3). The four expected tryptic peptides (identified by amino acid analysis) of CB9 were obtained in their un-cross-linked form in the following yields: residues 84–100, 8%; residues 101–104, 31%; residues 105–120, 50%; residues 121–135, 19%. It seems unlikely, therefore, that residues in CB9 participate significantly in cross-linking to TnI. It is noteworthy, however, that the peptide which includes Cys-98 was once again found in lowest yield.

The peak of cross-linked material was divided into three fractions, X1–X3 (see Figure 3), each of which was sequenced. Each fraction showed the expected four sequences: CB8 and CN7 (which contain no Arg residues) plus TnI residues 104–112 and 138–152. No peptides from CB9 could be detected. CB8 was always present in the highest yield. The

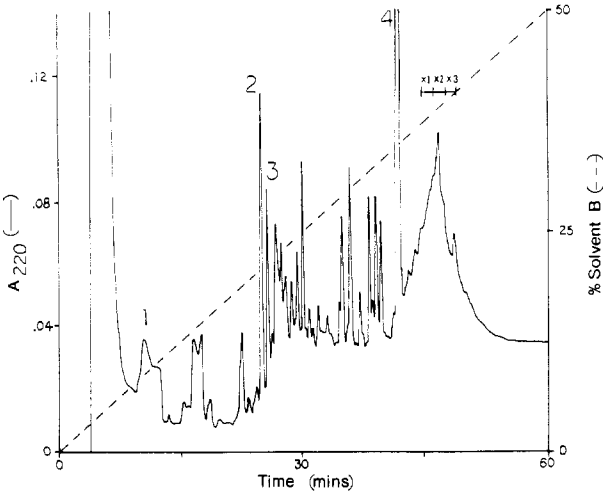

FIGURE 3: HPLC elution profile of the tryptic digest (restricted to Arg) of the cross-linked CNBr fraction (fraction X) shown in Figure 1. The separation was carried out on a Vydac 214TP54 (C_4) column. Numbered fractions designate tryptic peptides obtained from CB9 of TnC as follows: 1, 101–104; 2, 121–135; 3, 84–100; 4, 105–120. The broad, late-eluting fractions designated as X1, X2, and X3 contained cross-linked peptides (see text).

relative amounts of the three TnI sequences varied, but their total yield was always roughly equal to that of CB8 (see Table II). The TnI(104–112) sequence includes Lys-105 and Lys-107, but the yields of these two residues (despite contributions to the total Lys content from the other TnI peptides) were only about half the amounts that would be expected from the un-cross-linked peptide. This was confirmed by comparison with an independent sequence analysis of un-cross-linked, homogeneous TnI(104–112). These results indicate that Lys-105 and Lys-107 of TnI are cross-linked to the CB8 region of TnC. Due to the complexity of the sequencer results and the large number of acidic residues in CB8 (11, each of which may be cross-linked), it was not possible to identify individual cross-linked residues in TnC.

TnC×I was also directly digested with trypsin after citraconylation of the lysine residues. HPLC (not shown) again yielded a late-eluting, broad fraction which was not seen in a control digest of un-cross-linked TnC and TnI. Sequence analysis of this fraction yielded very complex results, revealing as many as seven simultaneous sequences. The highest yielding and most easily identified sequence was that of the peptide TnC(45–81), which is nearly coterminus with CB8 and, like CB8, was not recovered from a control tryptic digest of un-cross-linked TnC. This peptide, whose acid-insoluble nature has been noted previously (Ingraham & Hodges, 1988), was, like CB8, not recovered from the tryptic digest of un-cross-linked TnC. These results provide further evidence that peptides derived from this region of TnC are solubilized by cross-linking to TnI. The yields of two tryptic peptides from the CB9 region of TnC (residues 82–100 and residues 121–135) were about 17% and 30%, respectively, of the yields that were obtained from a digest of un-cross-linked TnC.

In another attempt to identify sites of cross-linking, SAP digestion of TnC×I was carried out in the presence of 4 M urea (Tarr, 1986). As before, an equimolar mixture of TnC and TnI was digested under identical conditions. HPLC profiles of the two digests (Figure 4) were very similar, with the now familiar exception of a late-eluting, broad fraction found only in the TnC×I digest. This cross-linked fraction yielded three major sequences, derived from expected cleavage at a limited number of Glu residues, which overlapped CB8 and CN4 (see Table II). It was not possible to accurately

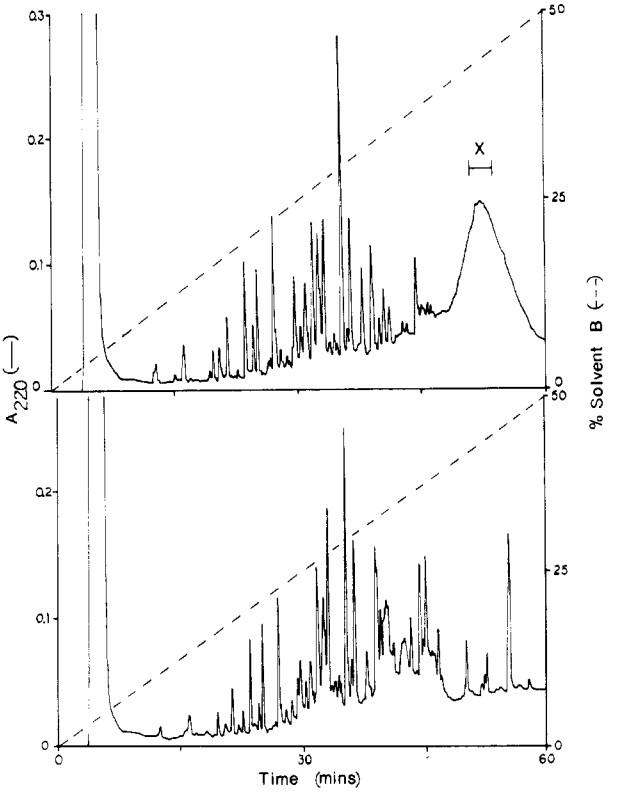

FIGURE 4: HPLC elution profile of SAP digests of TnC×I (upper panel) and an equimolar mixture of TnC and TnI (lower panel). Approximately 7 nmol of digested protein was injected. separations were carried out on Vydac 218TP54 (C_{18}) column. The late-eluting broad fraction (designated by an X) in the digest of TnC×I contained crosslinked peptides (see text).

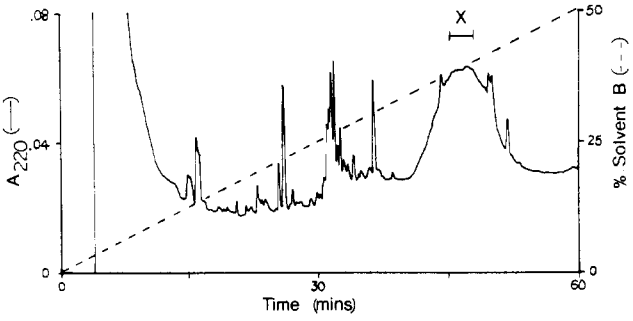

FIGURE 5: HPLC elution profile of the CNBr digest of the cross-linked SAP fraction (fraction X) shown in Figure 4. The separation was carried out on a Vydac 218TP54 (C_{18}) column. The broad, late-eluting fraction (designated with an X) contained cross-linked peptides (see text).

determine the yield of TnI Lys-105 in the SAP cross-linked fraction due to the contribution from the TnC sequences. TnI Lys-107, however, occurred in reduced yield. Again, the yields of the acidic amino acids of TnC were difficult to quantify because of the complexity of the results. In contrast to the results obtained from the CNBr and tryptic digests, with SAP the total yield of the two TnC sequences was much higher than that of the single TnI sequence. This may be due to the presence of many low-yield TnI peptides, derived from partial cleavage, whose sequences could not be identified. No sequences from the CB9 region of TnC could be detected.

The SAP cross-linked fraction was digested with CNBr, and a late-eluting, broad fraction was again observed upon HPLC analysis (Figure 5). This fraction yielded five identifiable sequences, all of which were consistent with our other results (see Table II). It was not possible to identify specific cross-

linking sites due to the complexity of the sequencer analysis.

Discussion

Interpretation of the present results requires consideration of some aspects of the zero-length cross-linking method adopted for these studies (Grabarek & Gergely, 1988). We used a two-step procedure to form the TnC×I cross-linked product. EDC and NHS were used to form active NHS esters of Asp and Glu side chain COOH groups in TnC. An equimolar amount of TnI was then added, under conditions which favor the formation of a native complex between the two proteins. Side chain Lys NH_2 groups from TnI reacted to form zero-length, isopeptide cross-links with activated COOH groups on TnC. If no amino groups were nearby, the NHS esters would slowly hydrolyze to regenerate the original TnC COOH groups. An advantage of zero-length cross-linking, as compared to site-directed cross-linking, is the lack of specificity in the sense that any COOH group can be activated and if it is close to a lysine side chain a cross-link, indicative of their interaction in the native complex, may form. On the other hand, hydrophobic interactions, or ionic interactions between Arg and Glu or Asp, are not detected. It must also be kept in mind that internal TnC cross-linking could occur, for example, if Lys and Glu or Asp acid residues were located in consecutive turns of an α-helix. A single molecule of TnC×I may therefore contain both intramolecular and intermolecular cross-links.

The CNBr, tryptic, and SAP digests of TnC×I all yielded late-eluting, broad HPLC fractions containing cross-linked peptides. The chromatographic behavior of the cross-linked peptides may be attributed, at least in part, to their heterogeneity Another important characteristic of these fractions is that they all contain peptides derived from the CB8 region of TnC. Such peptides were not recovered from tryptic and CNBr digests of un-cross-linked TnC because they were insoluble in the acidic HPLC solvents used in this study. Our results strongly indicate that cross-linking to TnI has altered the solubility of the TnC peptides, allowing their recovery from HPLC. The unusual solubility properties of the CB8 region of TnC may have contributed to the diffuse nature of the cross-linked HPLC fractions. TnI peptides spanning residues 92–167 were also found in the cross-linked fractions. Of these TnI peptides, CN4 (residues 96–116) and a peptide derived from it (residues 104–112) were obtained in the highest yields, and TnI Lys-105 and -107 were specifically identified as cross-linked residues.

The present data, then, unambiguously show the formation of zero-length cross-links between the CB8 region of TnC (residues 46–78) and the inhibitory CN4 region of TnI (residues 96–116). Some additional cross-linking occurs in TnI segments spanning residues 122–152, suggesting that the interaction site extends past the inhibitory region. The involvement of CB8 in cross-linking is consistent with the earlier conclusion that the negatively charged "helix C", which corresponds to the N-terminal part of CB8, represents one of the sites of interaction with TnI (Garbarek et al., 1981). Furthermore, the involvement of segment 122–152 of TnI is in agreement with the data of Dobrovol'sky et al. (1984) showing that Cys-133 of TnI can be cross-linked to Cys-98 of TnC.

The finding that the major reaction partner of CB8 is the CN4 segment in TnI was rather surprising in light of the well-established interaction of CN4 with the CB9 region of TnC. Previous studies suggested that CB9, or more specifically segment 89–100 (a part of CB9 which includes Cys-98), interacts with CN4 (Weeks & Perry, 1978; Dalgarno et al., 1982; Cachia et al., 1983; Leszyk et al., 1987, 1988). We found no evidence, however, for cross-linking of TnI with the CB9 region of TnC. The presence of CB9 in the CNBr cross-linked fraction must have been due to simple coelution (see Figure 2), since cross-linked fractions obtained after tryptic and SAP digestion contained no CB9-derived peptides.

The formation of internal cross-links, which has recently been demonstrated for TnC (Grabarek et al., 1989), may have interfered with cross-linking between TnI and the 89–100 segment of TnC. The Glu side chains at positions 92, 93, and 94 of TnC, implicated in the interaction with TnI (Grabarek et al., 1981), are, owing to the helical conformation of this segment, close to Lys residues at positions 88 and 90 and could undergo intramolecular cross-linking prior to the addition of TnI. In support of this idea, it is noteworthy that un-cross-linked tryptic peptides containing residues 89–100 were obtained in especially low yields.

The apparent absence of cross-linking involving the N-terminal segment of TnI, another putative site of interaction with TnC (Syska et al., 1976), is a noteworthy result of the present study. The NH_2-terminal TnI peptides CN5 (residues 1–22) contains two Lys at positions 5 and 18 and four Arg at positions 6, 8, 13, and 14. The lack of cross-linking in this region indicates that ionic contacts with TnC (if indeed this is an interaction site) would be predominantly made by Arg. Such an interpretation agrees with the ^1H NMR data of Grand et al. (1982), which showed that the resonances of eight amino acid residues in the middle of CN5, including the four Arg but neither of the Lys, were perturbed upon interaction with TnC.

The cross-linking between CB8 and CN4 documented here raises the question of the physiological importance of this interaction. While CB8 comprises Ca^{2+}-binding site II—one of the two sites implicated in triggering the conformational changes leading to the activation of the actomyosin machinery—neither this fragment in itself nor the fragment containing the entire NH_2-terminal domain (residues 9–84) of TnC can mimic the activating properties of the whole molecule (Grabarek et al., 1981). A slightly longer fragment, comprising residues 1–100, has approximately 30% of the activity of the intact molecule when substituted for TnC in the regulated actomyosin complex. The C-terminal fragment (residues 89–159) has similar activity. These data indicate that, whereas segment 89–100 is necessary for activity, it is not sufficient by itself and some additional interactions involving both halves of the TnC molecule must play a role.

While the present data provide evidence for the interaction between CB8 and CN4, they do not negate the interaction between CB9 and CN4 established by other means. If cross-link formation between CB8 and CN4 reflects additional interacting sites between TnI and TnC, one has to ask how both CB8 and CB9 could simultaneously interact with the inhibitory segment of TnI. This would be unlikely if TnC were to maintain the extended structure derived from the X-ray crystallographic studies (Sundaralingam et al., 1985; Herzberg & James, 1985). However, if evidence derived from low-angle X-ray scattering (Heidorn & Trewhella, 1988) and fluorescence energy transfer (Wang et al., 1987; Cheung & Wang, 1989) suggesting a more compact structure of TnC in solution applies to the in situ situation, simultaneous interaction of the segments corresponding to CB8 and CB9 with the inhibitory region of TnI may be possible.

The possibility of a folded structure of TnC having biological significance gains added support from the recent work of Persechini and Kretsinger (1988a) using a genetically engineered variant of calmodulin with Cys residues introduced into

its two domains at positions 3 and 146, respectively. It was possible to cross-link the two Cys via a disulfide bridge, indicating that the distance between them in solution must be shorter than the 37 Å indicated by the X-ray structure (Babu et al., 1985). The cross-linked mutant calmodulin was able to activate myosin light chain kinase, even after cleavage of the central helix with trypsin. Such cleavage destroys activity in the absence of cross-linking or in the wild-type calmodulin (Newton et al., 1984), so these results were taken to indicate that the contributions of both domains are necessary for activity. The authors proposed a model in which a segment of myosin light chain kinase is docked between the two domains of calmodulin, connected in a flexible way by the central helix (Persechini & Kretsinger, 1988b).

Our present cross-linking results are consistent with a similar model for the interaction between TnC and TnI. It should be emphasized, however, that in this context calmodulin should be taken merely as a model. Further experiments, particularly in a fully regulated actin system, will be needed to establish the mode of three-dimensional interactions among the regulatory and regulated components.

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