Photo-Cross-Linking of Rabbit Skeletal Troponin I Deletion Mutants with Troponin C and Its Thiol Mutants: The Inhibitory Region Enhances Binding of Troponin I Fragments to Troponin C[†]

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ABSTRACT: Contraction of vertebrate striated muscle is regulated by the strong Ca^{2+}-dependent interaction between troponin I (TnI) and troponin C (TnC). To critically evaluate this interaction, we generated four recombinant deletion fragments of rabbit fast skeletal TnI: the NH_2-terminal fragment (TnI_{1-94}), the NH_2 terminus and the inhibitory region (TnI_{1-120}), the inhibitory region and the COOH terminus (TnI_{96-181}), and the COOH-terminal fragment ($TnI_{122-181}$) containing amino acid residues 1−94, 1−120, 96−181, and 122−181, respectively. Native TnC and seven thiol mutants, containing single cysteine residues in the two globular domains and in the central helix of TnC, e.g., Cys-12, Cys-21, Cys-57, Cys-89, Cys-122, Cys-133, and Cys-158, were labeled with 4-maleimidobenzophenone, and their interaction with the recombinant TnI fragments and the synthetic inhibitory peptide (TnI_{98-114}, residues 98−114) was studied by photo-cross-linking. Extensive cross-linking occurred between various domains of TnC and TnI. The cross-linking patterns (a) showed that both NH_2- and COOH-terminal fragments of TnI are accessible to both of the globular domains of TnC, (b) indicated that linkage of the NH_2- and COOH-terminal sequences to the inhibitory region of TnI (TnI_{ir}) caused marked enhancement of cross-linking with native TnC and all seven thiol mutants, and (c) identified the region in TnC where TnI_{ir} binds as that containing residues 98, 133, 158, and 57. Thus, the results suggest that TnI and TnC may adopt flexible and dynamic conformations in which multiple interactions involving various domains of the two polypeptides occur and TnI_{ir} acting as a linker facilitates these interactions. The interaction of TnI and its fragments with actin, TnC, and TnT, considered together with the biological activity indicates that residues 96−120 represent a key structural and functional region of TnI. Whereas the NH_2-terminal region of TnI stabilizes binding to TnC and TnT, the COOH-terminal region stabilizes TnC and actin binding.

The troponin (Tn)[1] complex, a thin filament myofibrillar protein, is involved in the Ca^{2+}-regulated contraction of vertebrate striated muscles. The three structurally and functionally different subunits of Tn are troponin C (TnC), troponin I (TnI), and troponin T (TnT). TnC binds Ca^{2+}, TnT attaches the Tn complex to tropomyosin (Tm), and TnI is the inhibitory subunit which binds to the actin−Tm and inhibits actin−myosin interaction in the absence of Ca^{2+}, thereby preventing muscle contraction. The binding of Ca^{2+} to TnC triggers muscle contraction through a process of "information transfer" that is propagated by protein−protein interactions and conformational changes in virtually all thin filament proteins [for reviews, see Leavis and Gergely (1984), Zot and Potter (1987), and Farah and Reinach (1995)]. The key event in this regulatory process is the Ca^{2+}-induced change in the interaction between TnC and TnI. Although the interacting regions of TnC and TnI are tentatively identified, the precise structure of the binary complex, the conformational changes of these two Tn subunits upon Ca^{2+} binding, and the role of specific domains of the two protein subunits in binary interaction remain to be elucidated.

TnC belongs to the superfamily of Ca^{2+} binding proteins which has the so-called EF-hand structure [for a review, see Nakayama and Kretsinger (1994)]. The crystal structure of turkey and chicken TnC has shown that it is a dumbbell-shaped molecule with two globular domains connected by a single 31 residue long α-helix (Herzberg & James, 1985; Sundarlingam et al., 1985). Two low-affinity Ca^{2+} binding sites (I and II) and two high-affinity Ca^{2+}−Mg^{2+} binding sites (III and IV) are located in N- and C-terminal domains of TnC, respectively. The binding of Ca^{2+} to sites I and II

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[1] Abbreviations: BP-Mal, 4-maleimidobenzophenone; Tn, troponin; TnC, troponin C; TnI, troponin I; TnI_{1-94}, TnI_{1-120}, TnI_{96-181}, and $TnI_{122-181}$, recombinant TnI fragments containing amino acid residues 1−94, 1−120, 96−181, and 122−181, respectively; TnI_{98-114}, synthetic TnI inhibitory peptide containing amino acid residues 98−114; TnI_f, fast skeletal TnI; TnI_{ir}, inhibitory region of TnI; TnI_w, wild-type TnI; TnT, troponin T; Tm, tropomyosin; P-TnC^{98}, P-TnC^{12}, P-TnC^{21}, P-TnC^{57}, P-TnC^{89}, P-TnC^{122}, P-TnC^{133}, and P-TnC^{158}, TnC with attached BP-Mal probe at amino acid residues Cys-98, Cys-12, Cys-21, Cys-57, Cys-89, Cys-122, Cys-133, and Cys-158, respectively; PCR, polymerase chain reaction, UTR, untranslated region; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; βME, 2-mercaptoethanol; IOD, integrated optical density; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; S1, myosin subfragment 1.

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of TnC triggers the muscle contraction. TnC contains nine helical segments which are referred to as N-terminal helix, followed by helices A−H. Ca^{2+} binding to sites I and II causes the movement of helices B and C away from helices A and D which opens a hydrophobic cavity. A similar cavity is formed near sites III and IV also. These cavities appear to be the sites of interaction with TnI [for a review, see Grabarek et al. (1992)].

TnC and TnI interact at multiple regions, and the interaction takes place in both the presence and absence of Ca^{2+}. Studies using proteolytic fragments of TnC suggested that three regions of TnC bind to TnI: residues 50−60 (C-helix) and 89−100 (E-helix) which are Ca^{2+} dependent and residues 126−136 (G-helix) which is Ca^{2+} independent [for a review, see Zot and Potter (1987)]. Cyanogen bromide fragments of TnI containing residues 1−21 and 96−116 bind to TnC (Syska et al., 1976). Residues 96−116 bind either to TnC or to actin and inhibit actomyosin ATPase. Using synthetic peptides, Talbot and Hodges (1981) found that residues 104−115 represent the essential minimum sequence for the inhibitory activity of TnI, although it was less active than intact TnI. These studies indicated the functional importance of other TnI regions in interaction with Tn subunits and actin−Tm. Comparative sequence analysis of the vertebrate TnI intra- and interspecies isoforms and their cDNAs indicates that the C-terminal halves of TnI isoforms, including the inhibitory peptide (residues 101−182), show a high level of sequence conservation whereas the N-terminal halves (residues 1−100) show considerable divergence in sequence and size (Wu et al., 1993). These features may have significant relevance to genomic evolution and biological function of members of the vertebrate TnI multigene family.

Two models for TnI−TnC interaction have been proposed recently. Farah et al. (1994) studied the TnI−TnC interaction in gel assays using the deletion mutants of chicken skeletal TnI and deletion and site-directed mutants of TnC. They found that TnI can be divided into structural (N-terminal half) and regulatory (C-terminal half) regions which interact in an antiparallel fashion with the corresponding structural (C-terminal domain) and regulatory (N-terminal domain) regions of TnC. The inhibitory region of TnI (TnI_{ir}) appears to interact with both domains. Olah and Trewhella (1994) studied TnI−TnC interaction of intact molecules, with all four Ca^{2+} binding sites of TnC occupied ($4Ca^{2+}$·TnC), by small-angle X-ray and neutron scattering. Their results support a model in which $4Ca^{2+}$·TnC has an elongated dumbbell configuration akin to that in the crystal structure of TnC and TnI appears to be a regular right-handed spiral winding from one end of TnC to the other end and extending beyond TnC at each end, suggesting that TnI and TnC as well as TnI−TnC, the binary complex, possess considerable conformational flexibility.

In this report, we have selected photo-cross-linking to study the interaction of TnC with rabbit fast skeletal TnI (TnI_f) and its deletion mutants containing the combination of conserved and variable sequences. Native TnC and seven monocysteine mutants of TnC (in which the native Cys-98 is replaced by Leu) were labeled with the sulfhydryl-specific photo-cross-linker 4-maleimidobenzophenone (BP-Mal) and then cross-linked to four recombinant deletion fragments of TnI, intact TnI, and the inhibitory peptide. Our results indicate that (a) extensive cross-linking occurs between various domains of TnI and TnC, (b) TnI_{ir} interacts with both globular domains and the central α-helix of TnC, (c) the linkage of TnI_{ir} facilitates and enhances the interaction of other regions, e.g., the N- and C-terminal fragments of TnI with TnC, and (d) TnI and TnC interact predominantly in antiparallel orientation, and both subunits may attain flexible and dynamic conformations during these multiple interactions. Our studies also strengthen the concept that amino acid residues 96−120 constitute the most important structural and functional region of TnI in terms of TnC, actin, and TnT binding. It is also shown that TnI_{ir} binds close to TnC residues 98, 133, 158, and 57.

MATERIALS AND METHODS

Construction of Plasmids Expressing TnI Deletion Mutants. Polymerase chain reaction (PCR) was used to generate the four recombinant deletion fragments of TnI. Rabbit TnI_f cDNA with 78 bp of the 5′ untranslated region (UTR) deleted (Wu et al., 1993) and cloned in a T7 promoter based vector pAED4 (Jha et al., 1994) was used as template for PCR amplifications. Primers complementary to pAED4 and/or TnI_f cDNA sequences were used to generate four deletion fragments of TnI: the N-terminal fragment TnI_{1-94}, encoding amino acid residues 1−94; the N-terminal and TnI_{ir}-containing fragment TnI_{1-120}, encoding amino acid residues 1−120; the C-terminal fragment $TnI_{122-181}$, encoding amino acid residues 122−181; and the C-terminal and TnI_{ir}-containing fragment TnI_{96-181}, encoding residues 96−181. These primers contained the *Bam*HI and *Nde*I sites for subcloning. PCR conditions were as previously described (Jha et al., 1994). The PCR product was treated with proteinase K and subcloned into *Nde*I−*Bam*HI sites of pAED4 and pET17b, another T7 promoter-based vector, by standard methods (Sambrook et al., 1989). The C-terminal fragment was also subcloned into the *Nde*I−*Bam*HI sites of a T7 based fusion vector (Ghosh & Lowenstein, 1996) in which there is an N-terminal 6X-histidine tag, followed by a site for cleavage by Kex2 protease (Brenner & Fuller, 1992). All constructs were characterized by restriction mapping and authenticated by complete bidirectional DNA sequencing.

Synthesis of Recombinant Proteins in Escherichia coli and Their Purification. For the expression of cDNAs encoding TnI_{1-94} and TnI_{1-120}, both subcloned in pAED4 and TnI_{96-181}-encoding cDNA subcloned in pET17b, *E. coli* strain BL21-(DE3)pLysS was used. The conditions for expression were as previously described (Jha et al., 1994; Wu et al., 1995) except that NZCYM medium (Sambrook et al., 1989) was used. The recombinant TnI_{1-94}, TnI_{1-120}, and TnI_{96-181} fragments were found mostly in the insoluble fraction of the *E. coli* lysate. The *E. coli* pellets from a 1 L culture of TnI_{1-94}, TnI_{1-120}, and TnI_{96-181} were sonicated in 50 mL of solution A [20 mM Tris·HCl, pH 7.5, 0.1 M NaCl, 1 mM DTT, 2 mM EDTA, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)] and centrifuged for 30 min at 27000*g*. The pellet was washed twice with solution A and once with solution B (20 mM Tris·HCl, pH 8.5, 50 mM NaCl, 1 mM DTT, and 2 mM EDTA), homogenized in 25 mL of solution C (6 M urea, 20 mM Tris·HCl, pH 8.5, 1 mM DTT, and 2 mM EDTA) for 1 h, and centrifuged at 100000*g* for 30 min. The supernatants containing TnI_{1-94} and TnI_{1-120} were purified to 90−95% purity by a second centrifugation at 160000*g* for 90 min. The supernatant of TnI_{1-94} was applied onto a DE-52 column [Whatman, 15 mL bed volume;

equilibrated with 6 M urea, 20 mM Tris·HCl, pH 7.5, 2 mM EDTA, and 10 mM 2-mercaptoethanol (βME)], washed with buffers containing 50 mM and 0.15 M NaCl, and then eluted stepwise with buffers containing 0.3 and 0.4 M NaCl. The supernatant containing TnI_{1-120} was loaded on a CM-Sepharose CL6B column (Sigma, 30 mL bed volume; equilibrated with 6 M urea, 50 mM sodium acetate, pH 5.2, 2 mM EDTA, and 10 mM βME), washed as above, and then eluted stepwise with buffers containing 0.15, 0.3, and 0.45 M NaCl. The supernatant containing TnI_{96-181} was loaded on a Bio-Rex-70 column (Bio-Rad, 15 mL bed volume; equilibrated with 6 M urea, 20 mM Tris·HCl, pH 7.2, 2 mM EDTA, and 10 mM βME), washed with buffer containing 0.1 M NaCl, and then eluted stepwise with buffers containing 0.25 and 0.38 M NaCl.

$TnI_{122-181}$ was synthesized as a fusion protein in *E. coli* strain SG100 and was found exclusively in the soluble fraction of the lysate. The pellet from a 1 L culture of $TnI_{122-181}$ was suspended in 40 mL of column binding buffer (5 mM imidazole, pH 7.0, 20 mM Hepes, pH 7.0, 0.1 M NaCl, and 25% glycerol) containing 1 mM PMSF, sonicated, and centrifuged at 100000*g* for 30 min. A prepacked 2.5 mL column of iminodiacetic acid (Sigma) was washed and equilibrated with (a) 25 mL of 100 mM EDTA, pH 8.0, (b) 25 mL of water, (c) 50 mL of $Ni(NO_3)_2$ in 20 mM sodium acetate, pH 5.2, (d) 25 mL of water, and (e) 25 mL of column binding buffer. The supernatant containing $TnI_{122-181}$ was loaded on the column, washed with 60 mM imidazole, pH 7.0, and eluted batchwise with 10 mL each of 125, 250, and 400 mM imidazole, pH 7.0.

The purity of purified recombinant TnI fragments was assessed by SDS−PAGE. Pooled fractions were dialyzed against 10 mM Tris·HCl, pH 7.2, and 10 mM DTT, lyophilized, and stored at −20 °C. The final yields of TnI_{1-94}, TnI_{1-120}, TnI_{96-181}, and $TnI_{122-181}$ were 15, 25, 40, and 30 mg L^{-1}, respectively, as quantitated by the method of Bradford (1976). The N-terminal 6X-His tag in purified $TnI_{122-181}$ was removed by incubation with Kex2 protease (Brenner & Fuller, 1992) in 40 mM Bis-tris, pH 7.0, 4 mM $CaCl_2$, and 0.1 M NaCl at 28 °C for 2 h using a 100:1 molar ratio of protein to enzyme.

Synthesis of TnI Inhibitory Peptide. The inhibitory peptide of TnI, represented by residues 98−114 and termed TnI_{98-114}, was obtained by solid-state peptide synthesis using amino acids whose α-amino groups were protected by the 9-fluorenylmethoxycarbonyl group. Cleavage from the resin and side-chain deprotection were carried out using 95% trifluoroacetic acid. The peptides were purified by reverse-phase HPLC.

TnC Mutants: Site-Directed Mutagenesis, Synthesis, and Purification. For generating monocysteine mutants, rabbit skeletal TnC residues S12 and A21 (A-helix), A57 (C-helix), G89 (E helix), S122 (F-helix), S133 (G-helix), and V158 (H-helix) were changed to Cys, and the native Cys (Cys-98) was replaced by Leu using in vitro mutagenesis as previously described (Wang et al., 1990; Kobayashi et al., 1991, 1994; Tao et al., 1995). The resulting mutants were termed TnC^{12}, TnC^{21}, TnC^{57}, TnC^{89}, TnC^{122}, TnC^{133}, and TnC^{158}, respectively. The mutant proteins were expressed in *E. coli* and purified as previously described (Wang et al., 1990; Kobayashi et al., 1991, 1994; Tao et al., 1995). The selected mutation sites are highly solvent exposed in the crystal structure of chicken skeletal TnC and are unlikely to affect the structural and functional properties of the protein (Tao et al., 1995).

Formation and Analysis of the Tn Complex and TnI-C Complex. TnI, TnC, and TnT were prepared from rabbit skeletal muscle as described by Potter (1982). For binary and ternary complex formation, an equimolar concentration of each subunit and TnI fragments was incubated at 28 °C for 90 min with gentle agitation and dialyzed extensively against (i) 3 M urea, 20 mM Hepes, pH 7.0, 0.15 M NaCl, 0.1 mM $CaCl_2$, and 4 mM DTT and (ii) 20 mM Hepes, pH 7.0, 0.1 M NaCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM DTT. The samples were subjected to electrophoresis through 8% polyacrylamide gels made in 25 mM Tris and 120 mM glycine, containing either 6 M urea or 10% (v/v) glycerol and either $CaCl_2$ (0.5 mM) or EDTA (5 mM). The reservoir buffer was the same as gel buffer but without urea or glycerol.

Cosedimentation of TnIs with Actin. TnI_w, TnI_{98-114}, and all other TnI fragments (3 μM) were mixed with F-actin in 1:1 molar ratio with or without Tm (0.43 μM) in F-actin buffer (2 mM Hepes, pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP, 50 mM KCl, and 2 mM $MgCl_2$). The binding of TnI fragments with actin was evaluated by their cosedimentation upon centrifugation at 340000*g* for 20 min at 4 °C. Identical volumes of unspun samples and centrifuged samples, partitioned into pellet and supernatant fractions, were subjected to 12% SDS−PAGE.

Western Blot Analysis. The protein components, separated on 8% polyacrylamide or 16.5% polyacrylamide−tricine−SDS gels (Schagger & Jagow, 1987), were transferred onto nitrocellulose and probed with antibody against rabbit TnI_f (Jha et al., 1993).

Acto−S1 ATPase Assay. The Tn complex was formed using rabbit muscle TnC, TnT, and muscle TnI (TnI_w) or TnI fragments in an equimolar ratio. The ATPase activity was determined in 10 mM imidazole, pH 7.0, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, and 4 mM ATP using actin:S1:Tm:Tn at a molar ratio of 7:2:1:1 (Jha et al., 1994).

Photo-Cross-Linking and Densitometry. The TnC monocysteine mutants were labeled with BP-Mal as described previously (Tao et al., 1986). Photo-cross-linking of labeled TnC (8 μM) and TnI or its fragments (10 μM) was carried out in a photochemical reactor (Rayonet RPR-100, Southern New England Ultraviolet, Hamden, CT). The proteins were irradiated in 20 mM Hepes, pH 7.5, 0.1 M NaCl, 0.1 mM $CaCl_2$, and 2 mM DTT at 4 °C for 20 min. DTT (10 mM) and EDTA (5 mM) were added to all the reaction mixtures before they were subjected to 12% SDS−PAGE. The cross-linked TnI−TnC bands were quantitated by densitometric scanning of SDS−polyacrylamide gels using Millipore Bioimage wholeband software (Millipore Inc., Bedford, MA). The integrated optical density (IOD), which takes into account the area and the optical density for each cross-linked band, was obtained. The IOD value for a given monocysteine TnC mutant and TnI_w cross-linked product was taken as 100%, and all other IOD values of the cross-linked product of various TnI fragments with the TnC mutant were then converted to a percent value. A difference of <15% in cross-linking among various mutants has been considered insignificant (within experimental error) in the interpretation of the results.

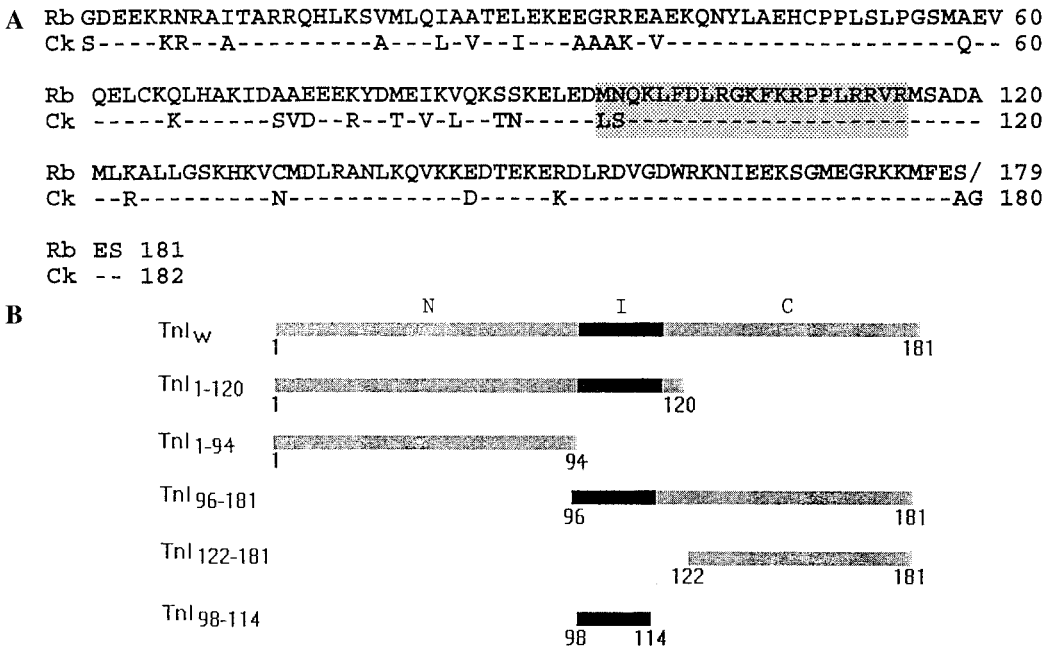

FIGURE 1: (A) Derived amino acid sequence of recombinant rabbit TnI_f (Rb) and its sequence comparison with recombinant chicken TnI_f (Ck). The Ck sequence is from Quaggio et al. (1993). TnI_{ir} is shaded. Identical residues are shown by dashes. (B) Schematic representation of various recombinant TnI deletion fragments and TnI_w. TnI_{ir} is designated I, and the sequences located on amino- and carboxy-terminal sides of this region are designated N and C, respectively. TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, $TnI_{122-181}$ (see also text) contain the N and I, N, I and C, and C regions, respectively. The number of amino acids at the beginning and end of each fragment is indicated. TnI_{98-114} containing amino acid residues 98−114 is a synthetic peptide representing the I region.

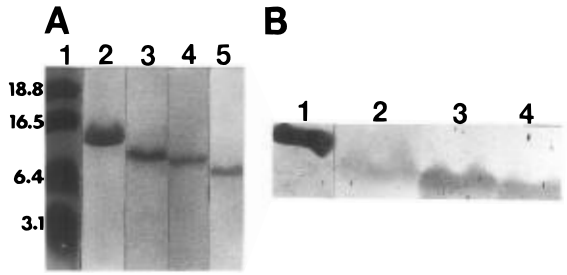

FIGURE 2: SDS−PAGE of purified recombinant TnI fragments. For details, see also Materials and Methods. (A) Lanes: 1, molecular mass markers with size (in kDa) indicated on the left; 2−5, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, and $TnI_{122-181}$, respectively. (B) Immunoblot using TnI_f antibody: lanes 1−4, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, and $TnI_{122-181}$, respectively.

RESULTS

Construction and Purification of Deletion Mutants. Complete bidirectional sequencing of mutant TnI_f cDNAs was carried out in order to obtain the derived amino acid sequence of the encoded TnI fragments used in this study. Since it is known that N-terminal methionine is cleaved off by *E. coli* in in vitro expressed intact as well as deletion mutants of chicken fast skeletal TnI (Quaggio et al., 1993; Farah et al., 1994), we have considered the various TnI deletion mutants to be authentic with N-terminal methionine deleted. The complete amino acid sequence of recombinant rabbit TnI_f and its comparison with chicken TnI_f, recently reported for deletion mutagenesis (Farah et al., 1994), is shown in Figure 1A. Various deletion mutants of rabbit TnI_f used in this study are schematically represented in Figure 1B. These fragments were purified as indicated in Materials and Methods, and their purity was assessed by SDS−PAGE (Figure 2A).

Biological Properties of TnI Fragments. All deletion TnI fragments (TnI_{1-94}, TnI_{1-120}, TnI_{96-181}, and $TnI_{122-181}$) and TnI_w cross-reacted with rabbit TnI_f antibody (Figure 2B), indicating that the entire TnI_f protein is immunogenic. TnC formed strong complexes of intermediate mobility with TnI_w, TnI_{1-94}, TnI_{1-120}, and TnI_{96-181} (Figure 3A) in urea gels in the presence of Ca^{2+} but not in the presence of EDTA (results not shown). The binary complex formed by TnI_{96-181} showed unusual slower mobility in this gel system. $TnI_{122-181}$ did not form a complex with TnC, whereas the complex formed by TnI_{98-114} was weak (Figure 3A). An almost identical pattern, as seen in the urea gel, was found in 10% glycerol gels. This nondenaturing gel system was used because it detects weaker interactions. $TnI_{122-181}$ did not form even a weak complex with TnC. The complex formed by TnI_{96-181} again showed an unusual slow migration (Figure 3B). Binary complexes formed by TnI_w, TnI_{1-94}, TnI_{1-120}, and TnI_{98-114} were stronger in glycerol gel as compared to urea gel, but surprisingly TnI_{96-181} formed a weaker complex in glycerol gel as compared to urea gel (compare panels A and B of Figure 3).

Immunoblot analysis of the ternary complexes revealed that only the complexes formed by TnI_w and TnI_{1-120} cross-reacted with this antibody as evidenced by the relative molecular sizes of the complexes (Figure 3C). In contrast, the acto−S1 ATPase assay showed Ca^{2+}-sensitive regulation for TnI_w and TnI_{96-181} and a very low level regulation for TnI_{1-120} (Table 1). The complex containing TnI_{96-181} showed nearly 80% Ca^{2+} sensitivity relative to TnI_w (Table 1).

The ability of various TnI fragments to bind actin in the absence of Ca^{2+} was tested by cosedimentation assay. The conditions were such that free TnI, or its fragments, would not sediment, but if they bind actin or actin−Tm, they would cosediment upon centrifugation. At a molar ratio of 1:1 of actin:TnI fragments, TnI_w, TnI_{1-120}, and TnI_{96-181} cosedimented with actin or actin−Tm, but TnI_{1-94} and $TnI_{122-181}$

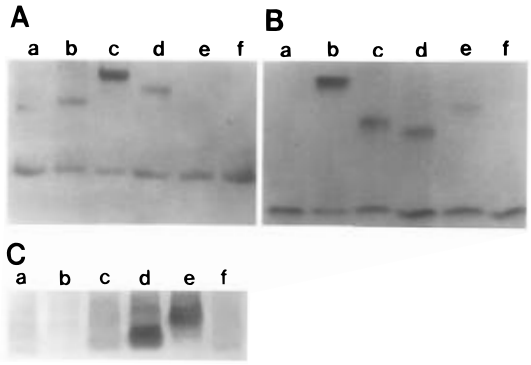

FIGURE 3: Ability of various TnI fragments to form binary (TnC−TnI) and ternary (TnC−TnT−TnI) complexes with rabbit skeletal TnC and TnT. For details, see Materials and Methods. The final concentrations of TnI_w, TnI_{1-94}, TnI_{1-120}, and TnI_{98-114} were 10.8 μM. For TnI_{96-181} and $TnI_{122-181}$, which are likely to form weak complexes, the concentrations were increased to 15.2 μM. (A) Binary complex formation with TnC in 6 M urea gels in the presence of 0.5 mM $CaCl_2$: lanes a−f, samples containing TnI_{1-94}, TnI_{1-120}, TnI_w, TnI_{96-181}, $TnI_{122-181}$, and TnI_{98-114}, respectively. Uncomplexed TnC is at the bottom of the gel. (B) Binary complex formation with TnC in 10% glycerol gel in the presence of 0.5 mM $CaCl_2$: lanes a−f, samples containing TnI_{98-114}, TnI_w, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, and $TnI_{122-181}$, respectively. Uncomplexed TnC is at the bottom of the gel. (C) Ternary complex formation with TnC and TnT. Immunoblotting of the complexes using rabbit TnI_f antibody is shown. Lanes a−f, samples containing $TnI_{122-181}$, TnI_{96-181}, TnI_{1-94}, TnI_{1-120}, TnI_w, and TnI_{98-114}, respectively.

Table 1: Regulation of the Acto-S1 ATPase Activity [mol of P_i (mol of S-1)$^{-1}$ min^{-1}] by Troponin Reconstituted with Various TnI Fragments[a]

troponin reconstituted with	ATPase activity		Ca^{2+} sensitivity (%)
	$+Ca^{2+}$	+EGTA	
no troponin	42.0	41.7	0.7
TnI_w (native TnI)	53.6	23.2	56.7
TnI_{1-94}	47.8	47.1	1.5
TnI_{1-120}	44.9	40.6	9.6
TnI_{96-181}	52.2	29.0	44.4
$TnI_{122-181}$	52.2	51.5	1.2

[a] The calcium sensitivity was calculated as follows: (1 − EGTA rate/Ca^{2+} rate) × 100.

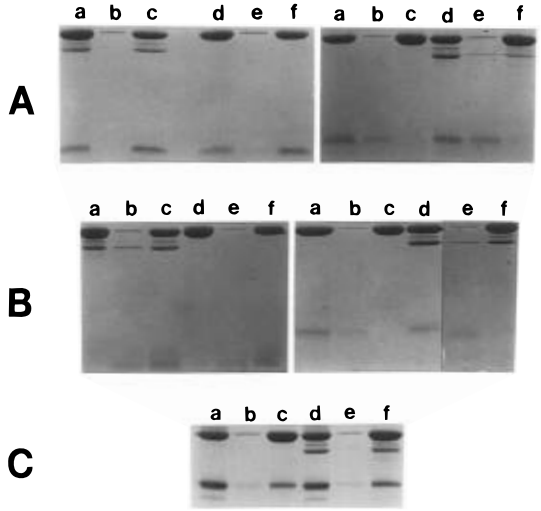

FIGURE 4: Cosedimentation of various TnI fragments with actin in the absence of Ca^{2+}. For details, see also Materials and Methods. Under the experimental conditions used, if actin and a TnI fragment interact, then they will cosediment in the pellet after centrifugation. In the absence of interaction, only actin will be pelletted and TnI will be in the supernatant. Experiments were carried out in the presence and absence of Tm. Equal volumes of unspun and spun samples containing the supernatant and pellet fractions separately of each reaction mixture were loaded in adjacent lanes. (A) Actin and recombinant fragments of TnI containing C-terminal regions. (Left panel) Incubations with TnI_{96-181}: lanes a−c, unspun, supernatant, and pellet, respectively, of reaction mixtures containing Tm; lanes d−f, unspun, supernatant, and pellet, respectively, of reaction mixtures without Tm. (Right panel) Incubations with $TnI_{122-181}$: lanes a−c, unspun, supernatant, and pellet, respectively, of reaction mixtures without Tm; lanes d−f, unspun, supernatant, and pellet, respectively, of reaction mixtures with Tm. (B) Actin and recombinant TnI containing N-terminal regions. (Left panel) Incubations with TnI_{1-120}: lanes a−c, pellet, supernatant, and unspun samples, respectively, with Tm; lanes d−f, pellet, supernatant, and unspun samples, respectively, without Tm. (Right panel) Incubations with TnI_{1-94}: lanes a−c, unspun, supernatant, and pellet, respectively, without Tm; lanes d−f, unspun, supernatant, and pellet, respectively, with Tm. (C) Incubations containing actin and TnI_w: lanes a−c, unspun, supernatant, and pellet, respectively, without Tm; lanes d−f, unspun, supernatant, and pellet, respectively, with Tm.

did not (Figure 4). Cosedimentation was more efficient with actin−Tm than actin alone. Also, whereas for TnI_{96-181} cosedimentation was complete and comparable to that of TnI_w, TnI_{1-120} cosedimented very poorly (Figure 4). TnI_{98-114} showed a cosedimentation pattern similar to that of TnI_{1-120} (result not shown).

Photo-Cross-Linking of TnI and TnC. The location of specific residues which were converted into cysteine in different monocysteine mutants of TnC is depicted in Figure 5. The mutant proteins were then labeled with BP-Mal. From here onward the native and mutant TnCs with attached BP-Mal probe are being referred to as P-TnC^{98}, P-TnC^{12}, P-TnC^{21}, P-TnC^{57}, P-TnC^{89}, P-TnC^{122}, P-TnC^{133}, and P-TnC^{158}. Irradiation of these TnCs with various TnI fragments resulted in the formation of a single photo-cross-linked product. The SDS−PAGE mobilities of the cross-linked products were consistent with the molecular mass of the TnC−TnI heterodimers (Figure 6) except for TnC−TnI_{98-114} heterodimers in some instances (Figure 6C, subpanel g). Irradiated uncomplexed P-TnCs formed one or more broad bands (Figure 6), which are most likely due to the formation of multiple intramolecular cross-linked products.

The cross-linked species obtained with various TnI fragments and the thiol mutants P-TnC^{98}, P-TnC^{133}, and P-TnC^{158} formed very sharp bands on SDS−PAGE. Somewhat moderate bands for P-TnC^{12} and P-TnC^{122} and broad weak bands for P-TnC^{21}, P-TnC^{57}, and P-TnC^{89} were observed (Figure 6). The TnI deletion fragments $TnI_{122-181}$ or TnI_{1-94}, especially the former, were not good substrates for cross-linking. Whenever TnI_{ir} was present in a TnI fragment, e.g., TnI_{1-120} and TnI_{96-181}, a high level of crosslinking was observed, suggesting that the presence of TnI_{ir} enhances the interaction between TnI and TnC. However, even without TnI_{ir}, considerable levels of cross-linking such as those with P-TnC^{21}, P-TnC^{133}, and P-TnC^{158} mutants and native TnC were observed (Table 2), indicating that other regions of TnI are also involved in TnI−TnC interaction. By comparison with other fragments, TnI_{98-114} was found to be a very good substrate for cross-linking with P-TnC^{57}, P-TnC^{133}, and P-TnC^{98} and a moderately good substrate for cross-linking with P-TnC^{158} (Table 2). This strongly suggests that the inhibitory peptide binds to both N- and C-domains of TnC close to residues 57, 98, 133, and 158. The above results also suggest that although TnI_{ir} strongly interacted with TnC,

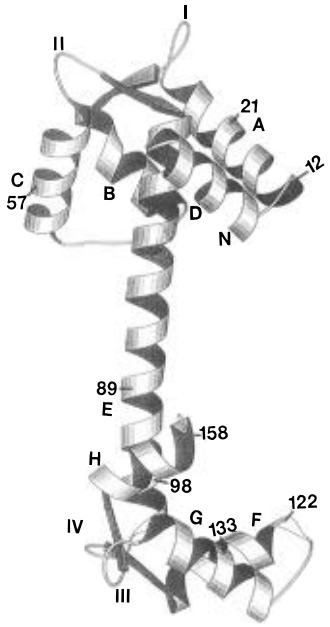

FIGURE 5: Ribbon model of TnC, adapted from Herzberg and James (1985), indicating the location of specific residues which were converted into cysteine (bold numbers) in different single monocysteine mutants. The nine helices termed N (N-helix) and A−H beginning from the NH_2 terminus of TnC are shown, and the four calcium binding sites, I−IV, are also indicated.

this interaction is strengthened if N- and C-terminal regions of TnI are also present.

Cross-linking of various TnI fragments with native TnC (P-TnC^{98}) and the central helix mutant, P-TnC^{89}, showed the following: P-TnC^{98}, located close to the C-terminal domain of TnC, cross-linked more efficiently to TnI_{96-181} than TnI_{1-120}, whereas P-TnC^{89}, which is closer to N-terminal domain of TnC, formed more cross-linked product with TnI_{1-120} relative to TnI_{96-181}. Contrary to this pattern, TnI_{1-94} showed considerably high cross-linking with P-TnC^{98}, while there was no cross-linked product with $TnI_{122-181}$. Overall, there was a decreased level of cross-linking of various TnI fragments and especially TnI_{98-114} with P-TnC^{89} as compared to P-TnC^{98}, indicating that the former residue is less exposed for TnI interaction. This may be due to the location of these residues on opposite sides of the central helix. Except for $TnI_{122-181}$, all other TnI fragments showed a high level of cross-linking with P-TnC^{98}, indicating that the important binding residues of TnI are located in the vicinity of Cys-98 of TnC. This is further supported by the high similarity in the cross-linking pattern of P-TnC^{98} with the inhibitory peptide (TnI_{98-114}) or TnI_{ir}-containing fragments TnI_{1-120} and TnI_{96-181}.

Cross-linking of various TnI fragments with three N-terminal mutants of TnC, P-TnC^{12}, P-TnC^{21}, and P-TnC^{57}, resulted in a single but weak cross-linked product (Figure 6A). However, on a relative basis, taking the TnC−TnI_w cross-linked product as 100%, the TnI fragments appeared to interact strongly with TnC, especially if they contained the TnI_{ir}. With P-TnC^{21}, the N-terminal fragments of TnI, TnI_{1-94}, and TnI_{1-120} gave higher cross-linking than the C-terminal fragments TnI_{96-181} and $TnI_{122-181}$. Among the three N-terminal TnC mutants, P-TnC^{12} showed the clearest and highest level of cross-linking with the various TnI fragments. Its cross-linking was greater with TnI_{96-181} than TnI_{1-120} (Figure 6A, subpanel b; Table 2). P-TnC^{57} cross-linked efficiently with TnI_{98-114} (Table 2) and also with TnI_{1-120} and TnI_{96-181}, both of which contain TnI_{ir}. The yield of cross-linking of P-TnC^{57} with TnI_{96-181} approaches that observed with intact TnI (Table 2). Taken together, these results suggest that (i) TnI_{ir}, whether linked to the N- or C-terminal region of TnI, shows a high level of cross-linking to N-terminal mutants of TnC and (ii) TnI_{ir} is closer to TnC^{57} than to TnC^{12} and TnC^{21} in a binary complex.

The C-terminal mutants of TnC showed 2−5-fold more cross-linking than the N-terminal mutants with various TnI

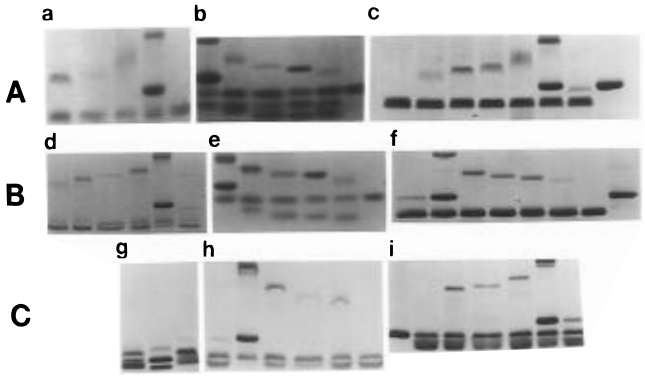

FIGURE 6: Photo-cross-linking of the BP-Mal probe attached to specific residues in monocysteine TnC mutants, with various TnI fragments. For details, see also Materials and Methods. (A) BP-Mal probe attached to cysteine residues located in N-terminal domain of TnC. (Subpanel a) P-TnC^{57}. Lanes (left to right): TnI_{96-181}, TnI_{1-94}, TnI_{1-120}, TnI_w, and $TnI_{122-181}$, respectively. (Subpanel b) P-TnC^{12}. Lanes (left to right): TnI_w, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, $TnI_{122-181}$ and non-cross-linked TnC^{12}, respectively. (Subpanel c) P-TnC^{21}. Lanes (left to right): non-cross-linked TnC^{21}, $TnI_{122-181}$, TnI_{96-181}, TnI_{1-94}, TnI_{1-120}, TnI_w, TnI_{98-114}, and non-cross-linked TnI_w, respectively. (B) BP-Mal probe attached to cysteine residues located in the C-terminal domain of TnC. (Subpanel d) P-TnC^{122}. Lanes (left to right): $TnI_{122-181}$, TnI_{96-181}, TnI_{1-94}, TnI_{1-120}, TnI_w, and TnI_{98-114}, respectively. (Subpanel e) P-TnC^{133}. Lanes (left to right): TnI_w, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, $TnI_{122-181}$, and non-cross-linked TnC^{133}, respectively. (Subpanel f) P-TnC^{158}. Lanes (left to right): TnI_{98-114}, TnI_w, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, $TnI_{122-181}$, non-cross-linked TnC^{158}, and TnI_w, respectively. (C) Cross-linking of the synthetic peptide TnI_{98-114} with various TnC thiol mutants (subpanel g) and cross-linking of various TnI fragments with TnC mutants containing cysteine residues located in the central α-helix (subpanels h and I). (Subpanel g) Lanes (left to right): TnI_{98-114} cross-linked to P-TnC^{57}, P-TnC^{12}, and P-TnC^{133}, respectively. (Subpanel h) P-TnC^{89}. Lanes (left to right): TnI_{98-114}, TnI_w, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, and $TnI_{122-181}$, respectively. (Subpanel i) P-TnC^{98}. Lanes (left to right): non-cross-linked TnC^{98}, $TnI_{122-181}$, TnI_{96-181}, TnI_{1-94}, TnI_{1-120}, TnI_w, and TnI_{98-114}, respectively.

Table 2: Photo-Cross-Linking of the BP-Mal Probe Attached to Different Monocysteine Mutant TnCs with Various TnI Fragments[a]

probe attached at TnC residue	% cross-linking				
	TnI_{1-94}	TnI_{1-120}	TnI_{96-181}	$TnI_{122-181}$	TnI_{98-114}
Cys-12	15	35	52	12	21
Cys-21	63	89	54	25	20
Cys-57	34	65	94	14	84
Cys-89	7	42	24	2	8
Cys-98	29	41	58	0	55
Cys-122	16	51	50	9	9
Cys-133	42	60	77	25	68
Cys-158	52	68	57	13	30

[a] The cross-linked products on SDS gels were densitometrically scanned. The values obtained for various TnI fragments were converted into percent by taking the cross-linked product formed by TnI_w on that particular gel as 100%. Two to four gels were scanned for each TnC mutant, and the mean values are shown.

fragments (Figure 6). P-TnC^{133} showed a somewhat higher yield of cross-linking with TnI_{96-181} as compared to TnI_{1-120}, although it showed a fairly high level of cross-linking with both N-terminal fragments of TnI, TnI_{1-94}, and TnI_{1-120} and also the inhibitory peptide, TnI_{98-114}. P-TnC^{158} showed more cross-linking with both N-terminal fragments of TnI and a much lower level of cross-linking with TnI_{98-114} as compared to P-TnC^{133} (Table 2). P-TnC^{122} cross-linked poorly with TnI fragments containing only the N- or C-terminal region, TnI_{1-94} and $TnI_{122-181}$, respectively, or the inhibitory peptide (TnI_{98-114}) (Table 2). Linkage of TnI_{ir} with either the N- or C-terminus (TnI_{1-120} and TnI_{96-181}, respectively) yielded a much higher and almost identical cross-linking value. Considered together, the cross-linking with three C-terminal mutants of TnC suggests that (i) TnI_{ir} linked with either N-terminal or C-terminal regions shows nearly the same level of cross-linking with these mutants, (ii) TnI_{ir} is closest to TnC^{133}, intermediate to TnC^{158}, and farthest from TnC^{122} in the complex, and (iii) their cross-linking to N-terminal fragments of TnI was more pronounced than to the C-terminal fragments of TnI.

DISCUSSION

As opposed to the proteolytic and synthetic peptides, which have been widely used in structure−function studies of the Tn subunits, the larger fragments of a protein better represent its native structure and often mimic the structure−function properties of the parent protein as has been shown for chicken skeletal TnI (Farah et al., 1994) and TnC (Pearlstone & Smillie, 1995). Furthermore, since TnI and TnC interact at multiple regions, generation of recombinant large deletion fragments of one protein and point mutations at selected target sites of another protein may offer an experimental approach to identify the interacting domains. Taking this approach, Farah et al. (1994) studied the interaction of truncated fragments of chicken TnI_f with native TnC and its mutations in Ca^{2+} binding sites by gel assays. One or more of the thiol mutants, used in our report, have also been used by other laboratories to study the cross-linking with native TnI only (Leszyk et al., 1987; Wang et al., 1990; Kobayashi et al., 1991, 1994; Tao et al., 1995). The TnI and TnC interactions have also been studied by diverse techniques [for a review, see Zot and Potter (1987)] including small-angle X-ray and neutron scattering (Olah & Trewhella, 1994). All these studies have yielded considerable valuable information on TnI and TnC interactions.

We have chosen photo-cross-linking of intact rabbit TnI_f and its various large truncated fragments containing the N- or C-terminal region with or without TnI_{ir} as the interacting entities, with native TnC and its seven monocysteine mutants containing CySH residues in both of the globular domains and in the central helix. This approach has enabled us to perform a comprehensive analysis of TnI−TnC interaction and to examine the role of a relatively conserved C-terminal half and a hypervariable N-terminal half, previously identified by us, which are present among the vertebrate TnI isoforms (Wu et al., 1993). In selecting the seven monocysteine mutants of TnC for cross-linking with different TnI_f fragments, we have taken into consideration the possibility that these mutations may alter the hydrogen-bonding pattern and van der Waals contact of amino acid side chains, resulting in modification of conformational dynamics between TnI and TnC, and thus affect the levels to which the proteins would cross-link. Therefore, we have selected TnC thiol mutants which (a) involved relatively conservative substitutions (see also Materials and Methods), (b) did not disrupt the binding of Ca^{2+} ions in N- and C-terminal domains, and (c) did not replace structurally and functionally important or highly conserved residues, e.g., four Phe residues in each globular domain which play a crucial role in forming the hydrophobic core of these domains (Sundaralingam et al., 1985; Herzberg & James, 1985, 1988). More importantly, none of the mutations appeared to be modifying the hydrogen-bonding pattern and van der Waals contact of side chains, based on the information available from the crystal structure of turkey TnC. For example, the side chains of the following paired residues, Asp36 and Thr72, Ile37 and 73, and Ile113 and 149 in turkey TnC (Herzberg & James, 1988), are known to be involved in van der Waals contact. The corresponding residues in rabbit TnC are 33, 69, 34, 70, 110, and 146, respectively, and none of these residues has been mutated in the present study. Also, the functional properties of TnC are not affected by labeling with BP-Mal, and the cross-linked species formed with TnI by these mutant TnCs most likely reflect true interactions between the two native proteins (Tao et al., 1986; Leszyk et al., 1987). Furthermore, cross-linking is not a result of random collision of TnI and TnC, as dissociating conditions do not allow binary cross-linking (Tao et al., 1986).

Since the construction of the various rabbit TnI_f fragments and their purification from the *E. coli* lysates are being reported for the first time, it was necessary to confirm their biological activities. A Ca^{2+}-dependent TnI−TnC binary complex formation was observed for almost all TnI fragments in urea and glycerol gels under our assay conditions (Figure 3A,B). However, the formation of the Tn complex by TnI_{1-120} but not by TnI_{1-94} or TnI_{96-181} (Figure 3C) suggests that TnI_{ir} together with N-terminal sequences is important for TnT and TnC interactions. In contrast, TnI_{ir} linked to the C-terminus of TnI (TnI_{96-181}) appeared to be primarily responsible for the Ca^{2+}-sensitive acto−S1 ATPase activity (Table 1) even though it did not form a Tn complex in gel assays with TnT and TnC. This strongly suggests that TnI_{96-181} binds to actin in the absence of Ca^{2+} and dissociates from it in the presence of Ca^{2+}. The N-terminus of TnI together with TnI_{ir}, but not the N-terminal region alone, mainly stabilizes the incorporation of TnT into a ternary complex even in the absence of actin−Tm, although it does not impart Ca^{2+}-sensitive regulation. These findings are consistent with the results of Pearlstone and Smillie (1985), which suggested that TnI residues 57−106 (part of the N-terminal region and part of TnI_{ir}) and TnT residues 197−250 have a heptad hydrophobic repeat capable of forming a coiled-coil structure with each other. Sedimentation analysis of actin and various TnI fragments showed that all proteins which cosedimented with actin contained TnI_{ir}. This is consistent with the view that TnI_{ir} interacts with actin. However, a much weaker binding of actin with TnI_{1-120} and TnI_{98-114} relative to TnI_{96-181} also suggests that the C-terminal sequence of TnI stabilizes binding of TnI_{ir} to actin−Tm. It appears then that whereas the highly conserved C-terminal half of TnI containing TnI_{ir} plays a critical role in the Ca^{2+} regulation of muscle contraction and actin binding, the variable N-terminal sequences in association with TnI_{ir} play a modulatory role in the interaction with other Tn subunits as reported by Farah et al. (1994) also. These

observations support the hypothesis that genomic sequences encoding the N- and C-terminal halves of vertebrate TnI isoforms have evolved in a divergent manner due to a differential functional demand on them.

Our cross-linking data are discussed by taking into consideration the published literature on this aspect. Probes attached to TnC[12], TnC[57], TnC[89], and TnC[98] have been shown previously to cross-link to TnI residues 132−141, 113−121, 108−113, and 103−111, respectively in native TnI (Leszyk et al., 1987; Kobayashi et al., 1991, 1994). Residues of TnI which cross-link specifically to TnC[21], TnC[122], TnC[133], or TnC[158] are not currently known (Tao et al., 1995). While our data are broadly consistent with the above findings, they also suggest a more extensive interaction of TnI with TnC. For example, native Cys-98 is known to cross-link to TnI residues 103−111 in the intact TnI (Leszyk et al., 1987). Thus, it is expected to cross-link with TnI_{96-181}, TnI_{1-120}, and TnI_{98-114} but not to TnI_{1-94} and $TnI_{122-181}$. We observed that P-TnC[98] did not cross-link to $TnI_{122-181}$. Also, it showed a high level of cross-linking to TnI_{1-120}, TnI_{96-181}, and TnI_{98-114}. Interestingly, it cross-linked to TnI_{1-94}, to which it was not expected to cross-link, at about 50% of that observed with TnI_{1-120} and TnI_{96-181}. The results expected of P-TnC[98] were also anticipated for P-TnC[89]. However, the latter cross-linked more efficiently with TnI_{1-120} than TnI_{96-181}, and its interaction with TnI_{1-94} and especially with TnI_{98-114} was extremely poor as compared to P-TnC[98]. In fact, among all the monocysteine TnC mutants and native TnC, P-TnC[89] showed the least cross-linking to all TnI deletion fragments and especially to TnI_{98-114} (Table 2). It appears that although P-TnC[89] cross-links to TnI residues 108−113 in the native protein (Kobayashi et al., 1994), it fails to do so with a short peptide constituting those residues, e.g., TnI_{98-114}. It is likely that some additional segments, perhaps toward the N-terminus, are required for efficient cross-linking of P-TnC[89] with TnI residues 108−113. It appears that although TnC[89] and TnC[98] and the surrounding region, e.g., residues 89−100, interact with TnI_{ir} [for a review, see Zot and Potter (1987)], the additional C- and N-terminal sequences, especially the latter, of TnI are involved in providing stability to these interactions (see also the following section).

Of the three N-terminal TnC mutants used herein, TnC[12] is known to cross-link to TnI residues 132−141 (Kobayashi et al., 1994). Thus, it is expected that TnI_{1-94}, TnI_{1-120}, and TnI_{98-114} would not cross-link to it, while TnI_{96-181} and $TnI_{122-181}$ would. Our results show that P-TnC[12] cross-linked to all the TnI deletion fragments (Table 2), although the percent of cross-linking to TnI_{96-181} was higher than to TnI_{1-120}. On the other hand, cross-linking to TnI_{1-94} and $TnI_{122-181}$ was much lower. The high level of cross-linking with TnI_{1-120} suggests that P-TnC[12] can cross-link to the TnI sequence upstream of residues 132−141 including TnI_{ir}. Another N-terminal thiol mutant, P-TnC[57], which cross-links to TnI residues 113−121 (Kobayashi et al., 1991) should cross-link to TnI_{1-120}, TnI_{96-181}, and possibly TnI_{98-114} but not to TnI_{1-94} and $TnI_{122-181}$. Our results are broadly consistent with the expected results, as there is a higher percentage of cross-linking of P-TnC[57] with TnI_{96-181} and TnI_{1-120} and a relatively much lower cross-linking with TnI_{1-94} and $TnI_{122-181}$. It is also expected that cross-linking to TnI_{96-181}, which includes residues 113−121 and sequences on either side, would be much higher as compared to TnI_{1-120}, and this turned out to be true. However, we also observed strong cross-linking of P-TnC[57] with TnI_{98-114} and considerable cross-linking with TnI_{1-94}, about 34%. This further strengthens our conclusion that the binding of TnI to TnC is extensive. Our results are in agreement with Leszyk et al. (1990), who showed the formation of zero-length cross-links between TnC residues 46−78 and TnI residues 96−116. These authors also reported that TnI residues 122−152 cross-linked to TnC residues 46−78, further supporting the flexible and extensive nature of TnI−TnC interactions involving multiple domains of the subunits. The large deletion fragments of TnI used in this report are likely to mimic intact TnI_f in the functional studies. However, the possibility that the use of fragments may have yielded a different result from that of the intact TnI used in previous study (Kobayashi et al., 1991, 1994) cannot be strictly excluded.

Several lines of evidence reported in the literature such as the binding of TnI and TnC mutants in native gels, cross-linking of intact TnI and TnC thiol mutants, NMR spectroscopy and selective isotope labeling of cardiac TnI and TnC, and complex formation of other EF-hand proteins with their target peptides [for details, see discussion in Kobayashi et al. (1994) and Krudy et al. (1994); for a review, see Farah and Reinach (1995)] support the antiparallel interaction of TnI and TnC. Analysis of a large number of cross-linking patterns reported in this study, in general, supports such an interaction. This is apparent in the case of P-TnC[12], P-TnC[57], and P-TnC[158]. Cross-linking patterns obtained with P-TnC[98], P-TnC[122], P-TnC[133], and P-TnC[158], which show that their interaction is more with the N-terminal fragment TnI_{1-94} than with the C-terminal fragment $TnI_{122-181}$ (Table 2), also support the antiparallel mode of interaction. Previously no cross-linking data had confirmed an interaction between the N-terminal region of TnI and the C-terminal region of TnC [for a review, see Farah and Reinach (1995)]. The use of three C-terminal thiol mutants, e.g., TnC[122], TnC[133], and TnC[158], has enabled us to show by crosslinking the preferred antiparallel interaction between the C-terminal region of TnC and the N-terminal region of TnI. It should be noted that using the large deletion TnI fragments we also observed several exceptions to the antiparallel interaction. For example, P-TnC[21], a N-terminal mutant of TnC, strongly cross-linked with both N-terminal fragments of TnI, TnI_{1-94}, and TnI_{1-120}, as well as the C-terminal fragment TnI_{96-181}. However, the cross-linked band with TnI_{1-120} was broad, indicating a heterogeneous product, whereas with TnI_{96-181} it was sharper, indicating a more homogeneous product (Figure 6A, subpanel c). Also, the higher level of cross-linking of (i) P-TnC[89] with TnI_{1-120} than with TnI_{96-181}, (ii) P-TnC[57] with TnI_{1-94} than with $TnI_{122-181}$, (iii) P-TnC[98] with TnI_{96-181} than with TnI_{1-120}, and (iv) P-TnC[133] with TnI_{96-181} than with TnI_{1-120} (Table 2) does not fit into the antiparallel mode of TnI and TnC interaction.

It appears that TnI_{ir}, whether linked to the N-terminal or the C-terminal fragment of TnI, enhances considerably the cross-linking with native TnC and its thiol mutants. This suggests that the N- and C-terminal regions of TnI in the recombinant fragments are close (∼10 Å, the length of BP-Mal) to and thus interact extensively with both globular domains of TnC. Interestingly, several recent reports have suggested that TnC exhibits considerable flexibility in conformation. Using the highly orientation sensitive mea-

surement of electron paramagnetic resonance, Li and Fajer (1994) showed that TnC exists in several different orientational conformations in the reconstituted muscle fibers. On the basis of tyrosine anisotropy decay studies on skeletal muscle TnC, Wang et al. (1993) suggested that TnC exists in a spectrum of conformations or exhibits dynamic fluctuations in solution. Extensive interactions between TnI and acetylated TnC based on zero-length cross-linking have been reported recently (Kobayashi et al., 1995). These observations lend support to a flexible and dynamic model of TnI−TnC interaction, wherein TnI can also adopt different configurations relative to TnC. All of the cross-linking patterns reported in this study indicated that multiple interactions involving various domains of the two subunits occur in a flexible manner. Furthermore, TnI_{ir} plays a key role in facilitating these interactions. It is quite likely that the dynamic fluctuations in conformation of both TnI and TnC (Li & Fajer, 1994; Wang et al., 1993; Kobayashi et al., 1995) may be responsible for the observed cross-linking patterns reported in this study.

With respect to the structural features which may account for the observed effect of TnI_{ir} in the cross-linking studies, it should be noted that TnI_{ir} is primarily α-helical and this configuration may be distorted in the middle by two proline residues forming a hairpin-like structure (Campbell & Sykes, 1991). Its ability to facilitate the enhanced interaction of the N- or C-terminal regions of TnI with all the TnC thiol mutants examined in this report may be explained, if the two domains of TnC are closer to each other in the TnI−TnC complex relative to the crystal structure of TnC (Kobayashi et al., 1991). Interestingly, a similar interaction of TnI_{ir} with TnC has been postulated (Grabarek et al., 1992, and references therein) on the basis of the molecular modeling studies of calmodulin, a protein which resembles TnC in structure and function, with its target proteins. Olah and Trewhella (1994) have proposed a model of the TnI−TnC complex in which both proteins adopt highly elongated structures. If this is the case, then TnI_{ir} must have an extended structure to span the distance between the two globular domains of TnC. Clearly, the three-dimensional structure of TnI_{ir} is needed to ascertain the nature and topography of the TnI_{ir}−TnC interacting interfaces.

Earlier investigations have suggested that the central helix of TnC, specifically residues 89−100, is the part of the binding site for TnI_{ir} (Grabarek et al., 1992; Farah & Reinach, 1995). Kobayashi et al. (1996) showed that the TnI peptide consisting of residues 104−115 binds to two sites in TnC: (i) Glu-60 and/or Glu-61 in the N-terminal domain and (ii) acidic residue(s) in segment 84−94 of the central α-helix. The cross-linking patterns obtained by using a large number of targeted CySH residues in various domains of TnC enable us to identify the most likely residues of TnC to which TnI_{ir} may bind in the binary complex. For example, the high level of cross-linking of various TnI fragments and the inhibitory peptide (TnI_{98-114}) with P-TnC^{133} and P-TnC^{158} in the C-terminus, with P-TnC^{98} in the central helix, and with P-TnC^{57} in the N-terminal region of TnC suggests that TnI_{ir} is in close proximity with these TnC residues during binary interaction. However, these residues also showed various degrees of cross-linking with the inhibitory peptide (TnI_{98-114}) suggesting that TnI_{ir} binds closer to residues TnC^{98} and TnC^{133} than TnC^{158} in the C-terminal region of TnC and to residue TnC^{57} in the N-terminal region of TnC. The binding site of TnI_{ir} in TnC, according to our results, appears to span a region consisting of residues 57, 98, 133, and 158 (see also Figure 5). This binding pattern is also consistent with the fact that the two hydrophobic pockets in the crystal structure of TnC (one each at the N- and C-termini, respectively) are the likely sites for TnI_{ir} binding [for a review, see Grabarek et al. (1992)].

Taken together with previously published results [for reviews, see Zot and Potter (1987), Grabarek et al. (1992), Farah et al., (1994), and Farah and Reinach (1995)], our observations on the biological properties of various TnI fragments and their interactions with TnT and TnC suggest that TnI residues 96−120, which bind to TnC in the presence of Ca^{2+} and to actin in the absence of Ca^{2+}, may be the most important structure−function region of TnI. TnI residues 96−120, as opposed to residues 96−116, may be the actin−TnC binding region, based on our cross-linking results and the actin−TnI cosedimentation experiments. This view is also supported by the work of Li et al. (1995), which has shown that the distance between TnC^{98} and a rabbit skeletal monocysteine TnI mutant (TnI^{117}) reduces from 40 Å, when Ca^{2+} sites I and II in TnC are empty, to 28 Å, when these sites are occupied by $2Ca^{2+}$. Additionally, residues ∼50−120 of TnI may also be involved in the binding of TnT, as suggested by Tn complex formation by TnI_{1-120} but not TnI_{1-94}.

In summary, photo-cross-linking studies of recombinant large deletion fragments of TnI_f with native TnC and its seven thiol mutants containing CySH residues in targeted domains indicate that extensive interactions occur between TnI and TnC. Whereas many of these interactions, in general, support an antiparallel orientation of the two subunits, all of the observed cross-linking patterns indicate that multiple domains of both polypeptides are involved in binary interaction. It is highly unlikely that these interactions occur in a static complex with well-defined rigid interfaces. Rather, our results favor the view that both TnI and TnC may have flexible and dynamic conformations. TnI_{ir} (residues 96−120), which is α-helical with a hairpin-like structure and which interacts with both globular domains of TnC, appears to be acting as a linker in enhancing considerably the interaction of other regions of TnI with various domains of TnC. Besides strengthening the concept that TnI_{ir} is primarily responsible for the actomyosin ATPase inhibitory activity of native TnI, our results show that it also plays an important structural role in facilitating the Ca^{2+}-induced binary TnI−TnC interaction. We also show that the site in TnC where TnI_{ir} binds spans a region containing residues 98, 133, 158, and 57 in the TnI−TnC binary complex, indicating that TnI_{ir} is capable of interacting with the central helix and two globular domains of TnC, although it binds more strongly to the C-terminal domain of TnC. This may account for the enhanced interaction of both N- and C-terminal region of TnI with TnC, when they are linked to TnI_{ir}. In addition to delineating the "localized" interactions involving different domains of TnI and TnC by cross-linking, these studies also provide an insight into the functional aspects of the highly conserved C-terminal region and the variable N-terminal region of TnI.

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