

Effects of Ca^{2+} and Subunit Interactions on Surface Accessibility of Cysteine Residues in Cardiac Troponin[†]

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ABSTRACT: Rabbit and bovine cardiac troponin (Tn) subunits and complexes were labeled with iodo- $[^{14}\text{C}]$ acetamide in the presence and absence of Ca^{2+} to determine the effects of tertiary and quaternary structure on exposure of Cys SH groups. This procedure serves both to map regions of subunit interaction and the effects of Ca^{2+} -induced conformational change and to indicate which Cys residues should be useful attachment sites for spectroscopic or cross-linking probes. After being labeled, Tn subunits were purified by using reversed-phase HPLC and subjected to tryptic cleavage with or without prior citraconylation. Cys-containing fragments were isolated by RP-HPLC, and the percent labeling was determined. Cys-75 and -92 of TnI were completely accessible to iodoacetamide both when TnI was labeled alone or when in the TnC-TnI complex. Both residues were largely inaccessible when Tn or the TnI-TnT complex was labeled, suggesting burial in the TnI-TnT interface. In contrast, the Cys from the N-terminal region of bovine TnT was stoichiometrically labeled when TnT was labeled alone, in native Tn or in a troponin-tropomyosin complex. Cys-35 and -84 of TnC are located in the nonfunctional Ca^{2+} binding loop I of cardiac TnC and helix D, respectively. For TnC alone, the percent labelings of Cys-35 and -84 were 11% and 26%, respectively (minus Ca^{2+}), and 16% and 63%, respectively (plus Ca^{2+}). For TnC labeled within Tn, the percent labelings of Cys-35 and -84 were 20% and 52%, respectively (minus Ca^{2+}), and 20% and 78%, respectively (plus Ca^{2+}). The Ca^{2+} -induced exposure of these residues, especially Cys-84, supports the Ca^{2+} -activated model of turkey skeletal TnC derived from crystallographic data [Herzberg, O., Moul, J., & James, M. N. G. (1986) *J. Biol. Chem.* 261, 2638].

Both cardiac and skeletal muscle contractions are initiated by Ca^{2+} binding to the regulatory protein troponin (Tn).¹ Tn consists of three subunits: TnC, the Ca^{2+} binding subunit; TnI, the inhibitory subunit; and TnT, the tropomyosin binding subunit. The complex is held together by noncovalent interactions between the three subunits. Current understanding of the topography of these interactions derives largely from work with skeletal muscle Tn involving four types of studies, for example, fragment binding (Syska et al., 1976; Grabarek et al., 1981; Pearlstone & Smillie, 1985), amino acid residue accessibility (Chong & Hodges, 1982a; Hitchcock-DeGregori, 1982), covalent cross-linking (Sutoh & Matsuzaki, 1980; Chong & Hodges, 1982b; Tao et al., 1986), and fluorescence energy transfer (Wang & Cheung, 1986; Leavis et al., 1986). The latter two techniques generally involve covalent modification of Cys residues with either a photoaffinity reagent or a fluorescent probe. This approach is useful because the limited number of Cys residues present enables labeling at specific known regions of the subunits.

The primary structures of the subunits of rabbit skeletal Tn have shown that RS-TnT has no Cys residues, RS-TnC has one (Cys-98) (Collins, 1974), and RS-TnI has three (Cys-48, -64, and -133). This contrasts with rabbit and bovine cardiac Tn where TnC has two Cys (35 and 84) (van Eerd & Takahashi, 1976; Wilkinson, 1980), TnI has two Cys (75 and 92)

(Grand et al., 1976) (corresponding to Cys-48 and -64 in the RS-TnI sequence), and TnT has either one (in BC-TnT) (Hincke et al., 1979; Leszyk et al., 1987) or none (in RC-TnT) (Pearlstone et al., 1986). Before one can devise a strategy for the incorporation of cross-linking reagents or other structural probes at the SH groups, one must know whether the SH groups are (1) involved in the sites of interaction between subunits of the TN complex, (2) buried within the tertiary structure of the individual subunits, or (3) exposed on the protein surface. Furthermore, it is useful to know how conformational changes induced by Ca^{2+} binding to TnC affect the degree of exposure of the various SH groups. This information can be obtained by labeling with iodo $[^{14}\text{C}]$ acetamide in benign media ($\pm\text{Ca}^{2+}$) of the individual cardiac Tn subunits, binary complexes, and native Tn. To determine which SH groups are labeled, the individual subunits are isolated using reversed-phase high-performance liquid chromatography (RP-HPLC) and the percentage labeling determined. In addition, for analysis of TnC, the subunit is citraconylated to limit cleavage to Arg residues and digested with trypsin, the fragments containing the two Cys residues, Cit 1 (1-46) and Cit 3 (84-102), are separated by using RP-HPLC, and the percent labeling of each SH group is

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¹ Abbreviations: Tn, troponin complex; TnC, troponin C; TnI, troponin I; TnT, troponin T; RS, rabbit skeletal; RC, rabbit cardiac; BC, bovine cardiac; α -TM, α -tropomyosin; TM, tropomyosin; TnCI, TnC-TnI binary complex; TnIT, TnI-TnT binary complex; Cit 1 through Cit 5, tryptic fragments of citraconylated cardiac troponin C (Cit 1 is 1-46, Cit 2 is 47-83, Cit 3 is 84-102, Cit 4 is 103-147, and Cit 5 is 148-161; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; RP-HPLC, reversed phase HPLC; AGTC, *N*-(4-azidobenzoyl)[2-³H]glycyl-S-(2-thiopyridyl)cysteine; CMC, (carboxymethyl)cysteine.

determined. Similarly, cardiac TnI and TnT were subjected to tryptic digestion and the appropriate fragments analyzed. However, this was done to determine the extent of nonspecific labeling of these subunits, rather than to differentiate between labeling levels of different Cys residues.

This general approach has been successful in our laboratory for RS-Tn (Chong & Hodges, 1982a). Due to the differences in location and numbers of Cys residues in cardiac Tn, we expect some significant differences in accessibility of SH groups which in turn should provide valuable information for understanding the quaternary structure of the Tn complex.

MATERIALS AND METHODS

BC-Tn was prepared from frozen heart tissue by using a modified version of the procedure of Strapans et al. (1972). RC-Tn was extracted from an acetone powder made from frozen rabbit hearts (Pel-Freeze) and purified according to a modified version of the procedure of Pato et al. (1981) in which all steps were conducted at 0 or 4 °C, and Tn was isolated as a precipitate using a 35–55% (NH₄)₂SO₄ cut. Both BC-Tn and RC-Tn were further purified by using hydroxyapatite chromatography (Eisenberg & Kielley, 1974), then dialyzed versus water, and lyophilized.

The Tn subunits and subunit fragments were separated and purified by using reversed-phase high-performance liquid chromatography with a Varian 5000 liquid chromatograph on either analytical (250 × 4.1 mm i.d.) or semipreparative (250 × 10 mm i.d.) SynChropak RP-P (C₁₈) or C₈ columns (SynChrom, Linden, IN). Tn was dissolved either in 0.1% trifluoroacetic acid (TFA) in water (solvent A) or in another aqueous solution such as the labeling buffers. Subunits were eluted by using a gradient constructed from solvent A and 0.05% TFA in acetonitrile (solvent B). The gradient program was as follows: linear gradient from 75% A/25% B to 55% A/45% B at 40 min, isocratic for 5 min, and then linear gradient to 50% A/50% B at 50 min. Troponins T, I, and C eluted at approximately 29% B, 35% B, and 41% B, respectively, on the semipreparative C₁₈ column used and at 38%, 41%, and 45%, respectively, on the analytical C₈ column used. The exact elution position depended on both the particular column used and the sample loading. Analytical columns were run at 1 mL min⁻¹ and semipreparative columns at 2 or 3 mL min⁻¹.

TnI tryptic fragments (75–93) were purified on a SynChropak C₈ analytical column as follows: 0–2 min isocratic at 100% A; 2–10-min linear gradient to 80% A/20% B; 10–70-min linear gradient to 50% A/50% B. TnI fragment (75–93) eluted as two distinct peaks for RC-TnI at approximately 27% B and 32% B, respectively.

Two labeled tryptic fragments from the N-terminus of BC-TnT were purified as follows: 0–5 min, 100% A; 5–10 min, linear gradient to 85% A/15% B; 10–55 min, linear gradient to 70% A/30% B. The minor (fragment 1) and major (fragment 2) CMC-containing peaks eluted at approximately 22% B and 23% B, respectively.

Citraconylated TnC tryptic fragments were isolated on a C₈ analytical column run at 0.5 or 1.0 mL min⁻¹ as follows: 0–1 min, 100% A; 1.1 min, 85% A/15% B; 1.1–21 min, linear gradient to 75% A/25% B; 21–25 min, linear gradient to 67% A/33% B; 25–75 min, linear gradient to 55% A/45% B. Radioactivity flow monitoring was performed by using a Radiomatic Flo-One Beta radioactive flow detector with Flo-Scint III as scintillant.

Labeling of Tn, Tn subunits, or complexes with iodo-[¹⁴C]acetamide (Amersham) was performed by using the general procedure for S-carboxamidomethylation of proteins

described by Chong and Hodges (1982a). Protein samples (4–6 mg/mL) were dialyzed vs 8 M urea, 50 mM Tris, pH 7.5, 2 mM EDTA, and 50 mM 2-mercaptoethanol to ensure reduction of all Cys residues, followed by dialysis against either the same solvent containing 1 mM dithiothreitol (DTT) instead of 50 mM 2-mercaptoethanol (for labeling of unfolded protein) or 50 mM Tris, pH 7.5, 1 M KCl, and 1 mM DTT for labeling under nondenaturing conditions. With the exceptions of the TnIT complex, samples to be labeled under nondenaturing conditions were next dialyzed vs 50 mM Tris, 0.3 M KCl, and 1 mM DTT, pH 7.5, containing one of the following: (A) 2 mM EDTA; (B) 3 mM MgCl₂/1 mM EGTA; (C) 1 mM CaCl₂. The final dialysis for the TnIT complex was 50 mM Tris, pH 7.5, 0.5 M NaCl, and 1 mM DTT. All dialysis solutions were sparged with N₂ and sealed with parafilm prior to dialysis. Following dialysis, spectrophotometric determination of oxidized DTT in the solution (Cleland, 1964) demonstrated that >90% of the DTT remained reduced. The samples were labeled for 30 min at room temperature with 4 mM iodoacetamide (giving an iodoacetamide to Cys plus DTT ratio of 2.7), and the reaction was quenched with excess reducing agent. The specific activity of the iodoacetamide used ranged from approximately 1000 to 2000 dpm/nmol. Following the isolation of the desired subunits or subunit fragment, the percent labeling was determined from amino acid analysis and scintillation counting of the hydrolysate. Variations in the percent labeling values of ±5% are not considered significant.

BC-TnC was citraconylated (Atassi & Habeeb, 1972) by the addition of citraconic anhydride (40-fold molar excess of citraconic anhydride to amino groups) to TnC dissolved in 0.2 M sodium phosphate, pH 9. The reagent was added in several small aliquots over a period of 1 h with constant magnetic stirring at room temperature. The pH was maintained between 9 and 10 by the addition of 3 N NaOH. Following the reaction, samples were dialyzed versus 50 mM NH₄HCO₃ adjusted to pH 9.0 with NaOH.

Tryptic digestions of TnI, TnT, and citraconylated TnC were performed at 37 °C for 16 h. Digestions of TnI and TnT were performed in 50 mM NH₄HCO₃/0.2 M KCl, pH 8.3, using a 1:25 weight ratio of TPCK-trypsin (Worthington) to protein whereas digestion of citraconylated TnC utilized a 1:100 molar ratio of trypsin to protein in 50 mM NH₄HCO₃, pH 9.0. Tryptic cleavages were quenched by freezing or addition of PMSF to 0.2 mM. Following tryptic digestion and quenching with PMSF, the TnC fragments were decitraconylated by stirring for 3 h at pH 1.8 at room temperature and then frozen until fragment isolation by HPLC. Chymotryptic digestions of labeled BC-TnT tryptic fragments were performed under the same conditions as the tryptic digestions.

Amino acid analyses were obtained with a Durrum D-500 amino acid analyzer after hydrolysis in 6 N HCl in evacuated sealed tubes for 24 h at 110 °C. Sequencing of labeled fragments of BC-TnT and RC-TnI was accomplished on an Applied Biosystems Model 470 A gas-liquid phase sequencer equipped with an Applied Biosystems PTH analyzer, Model 120 A.

Surface profile prediction plots were generated on an IBM-PC using a computer program described by Parker et al. (1986) supplied from S.P.I. Synthetic Peptides Inc. Department of Biochemistry, The University of Alberta.

RESULTS

RP-HPLC Purification of Tn Subunits. To simplify isolation of the Tn subunits for our study, a new purification procedure utilizing reversed-phase high-performance liquid

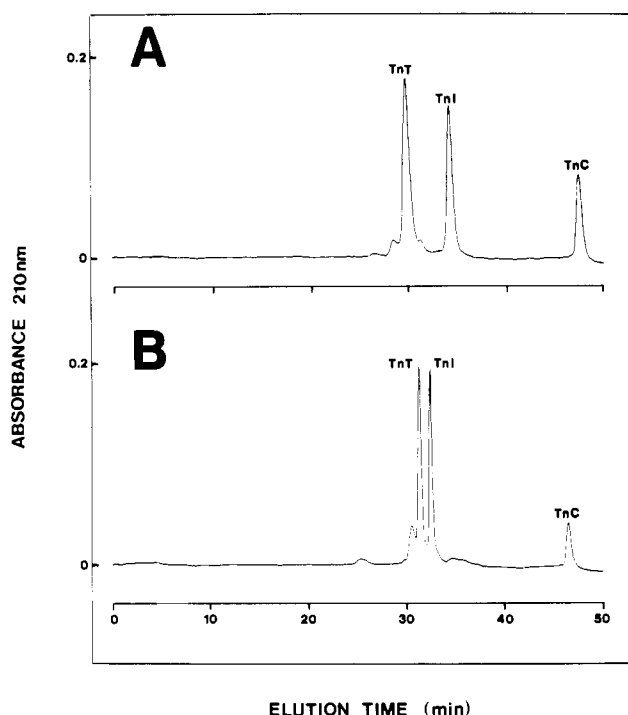


FIGURE 1: Separation of troponin subunits on an analytical C_8 RP-HPLC column. Panels A and B are the elution profiles for bovine cardiac troponin and rabbit skeletal troponin, respectively. Gradient conditions are described under Materials and Methods.

chromatography (RP-HPLC) was developed. The utility of this procedure for preparing small quantities (approximately 2–5 mg per run depending on whether analytical or semipreparative columns are used) of either cardiac or skeletal muscle Tn subunits is demonstrated in Figure 1, where the subunits of BC-Tn and RS-Tn are separated. The Tn subunits are applied to a C_8 and C_{18} reversed-phase column equilibrated with 75% solvent A (0.1% TFA/ H_2O) and 25% B (0.05% TFA/acetonitrile) and eluted with a gradient of increasing solvent B (0.5% B/min). The subunits elute in order of increasing hydrophobicity: TnT, TnI, and TnC. Although not shown in Figure 1, it is worthwhile to note that α -TM from rabbit heart nearly coelutes with cardiac TnI, following TnI by only half of 1% acetonitrile in the gradient. Consequently, use of this procedure for TnI isolation requires prior removal of TM from the Tn sample. Interestingly, neither Tn nor its subunits bound to a relatively nondenaturing hydrophobic-interaction HPLC column, the Bio-Gel TSK Phenyl-5PW column, equilibrated in a buffer containing 1.7 M $(NH_4)_2SO_4$ /0.1 M sodium phosphate, pH 7.0. Evidently, the surface hydrophobicity of the Tn subunits is insufficient to use this approach for Tn, its subunits, or binary complexes.

Previous work from our laboratory has demonstrated the usefulness of ion-exchange HPLC for purification of the isoforms of cardiac and skeletal muscle Tn subunits (Cachia et al., 1985). Similarly, using conventional ion-exchange chromatography, Gusev (1983) and Tobacman and Lee (1987) have separated two isoforms of BC-TnT. Although RP-HPLC does not appear to be as capable of separating isoforms as ion-exchange HPLC, the former method has advantages over both conventional ion-exchange chromatography and ion-exchange HPLC for routine work where isolation of single isoforms is not critical. First, urea is not used. Consequently, there is no danger of carbamylation of lysine residues due to cyanate formation. The other major advantage of RP-HPLC is that the solvent components used (water, acetonitrile, and trifluoroacetic acid) are all volatile, and lyophilization of the

Table I: Percent Labeling of the Cys Residue in Cardiac TnT with Iodo[^{14}C]acetamide

labeling conditions	BC-TnT ^a (%)	BC-TnT _{Tn} ^b (%)	BC-TnT _{Tn-TM}	RC-TnT _{Tn} ^d (%)
8 M urea	88			
EDTA		114		26
Mg ²⁺	99	113	114	21
Ca ²⁺		110		20

^aBC-TnT indicates the percent labeling of the individual subunit. ^bBC-TnT_{Tn} indicates subunit labeling within the Tn complex. ^cBC-TnT_{Tn-TM} indicates subunit labeling within the Tn-TM complex. ^dSince RC-TnT lacks any Cys residues, these values represent non-specific labeling of the RC-TnT subunit in the troponin complex and were calculated as percentage protein rather than percentage Cys labeled.

purified proteins can be performed immediately following separation, rather than following several dialysis steps. Previous work from numerous laboratories has shown that Tn subunits are not irreversibly denatured by high urea concentrations, suggesting that denaturation during RP-HPLC (Lau et al., 1984; Ingraham et al., 1985) should also be reversible. To determine if this is true for TnC, we examined the effect of Ca^{2+} on the far-UV circular dichroism spectrum of BC-TnC purified by using RP-HPLC. In the absence and presence of Ca^{2+} , $[\theta]_{222}$ values of 13 672° and 17 367° were measured, respectively. These values correspond to α -helical contents of approximately 51% in the absence of Ca^{2+} and 64% in its presence, indicating that the previously observed increase in α -helix upon Ca^{2+} binding (Burntrock et al., 1975) occurs in TnC purified according to our procedure.

Labeling of Cardiac TnT with Iodo[^{14}C]acetamide. RC-TnT has been sequenced and shown to consist of two isoforms, neither of which contains Cys (Pearlstone et al., 1986). BC-TnT also consists of two (Risnik et al., 1985; Tobacman & Lee, 1987; Leszyk et al., 1987) or possibly three (Cachia et al., 1985) isoforms. However, in the case of the two isoforms of BC-TnT which have been sequenced, both contain a single Cys residue, making this species of cardiac TnT potentially quite useful for physical studies of Tn (Leszyk et al., 1987). The major (TnT-1) and minor (TnT-2) BC-TnT isoforms described by Leszyk et al., 1987 have molecular weight values of 33 808 and 32 279, respectively, and their homologous Cys residues are located at positions 39 and 34, respectively, in the N-terminal regions of the molecules.

Table I displays the percentage labeling of rabbit and bovine cardiac TnT with iodo[^{14}C]acetamide. It is clear from these data that BC-TnT alone reacts quite well with the radioactive probe both in benign and in denaturing buffer solutions. Furthermore, when labeling of native BC-Tn occurs, the Cys residue appears to be completely labeled, both in the presence and in the absence of Mg^{2+} and Ca^{2+} ions. These results strongly suggest that the sulfhydryl group of BC-TnT is exposed on the surface of the molecule under all of these conditions. Since the NH_2 -terminal region of TnT has been implicated in the binding interaction between TnT and the head to tail overlap region of TM (Brisson et al., 1986), we also determined the percent labeling of BC-Tn within the Tn-TM regulatory complex. Significantly, no reduction in reactivity was observed (Table I), indicating that the TnT Cys residue is also exposed in the Tn-TM complex.

It appears that some non-Cys labeling of His, Met, or Lys residues, as described by Means and Feeney (1971), has occurred since superstoichiometric labeling of several of the BC-TnT samples took place with apparent percent labeling values of Cys residues ranging up to 114%. Furthermore,

Table II: Percent Labeling of Cys Residues in Cardiac TnI with Iodo[^{14}C]acetamide

sample	8 M urea (%)	labeling conditions		
		EDTA (%)	Mg $^{2+}$ (%)	Ca $^{2+}$ (%)
RC-TnI ^a			94	
RC-TnI _{CI} ^b		82	82	86
TnI _{Tn} ^c		7	8	8
fragment A (75-93)	93	0.5	0.6	0.9
fragment B (75-93)	97		0.6	0.7

^a RC-TnI indicates the individual subunit was labeled. ^b RC-TnI_{CI} indicates the subunit was labeled while in the TnC-TnI complex. ^c TnI_{Tn} is a summary of data from both RC-TnI and BC-TnI labeled in situ within the Tn complex.

20–26% of the RC-TnT molecules were labeled despite their lack of a Cys residue. In order to determine whether the BC-TnT labeling data in Table I were due primarily to specific labeling of the Cys residue, tryptic digestions of this subunit were performed and labeled fragments purified using RP-HPLC. As described under Materials and Methods, two radioactive fragments, 1 and 2, were obtained. Analyses of these fragments indicated similar amino acid compositions. Both fragments contain essentially stoichiometric levels of labeled CMC, with 83% of fragment 1 and 82% of fragment 2 being labeled, respectively. The high degree of similarity of these fragments and the presence in them of only one trypsin-sensitive site, an Arg residue, indicate that they derive from different isoforms of BC-TnT. Comparison of the compositions of these fragments to the terminal tryptic fragments of the isoforms of BC-TnT indicates that fragments 1 and 2 come from the TnT-2 and TnT-1 isoforms, respectively. This conclusion was confirmed by sequencing the C-terminal chymotryptic fragment derived from digestion of tryptic fragment 2 (cleavage at Tyr-10). Comparison of our sequence data starting at Glu-11 with those obtained by Leszyk et al. (1987) shows identity through Pro-38. Following Pro-38, ambiguity in determination of the identities of some of the residues occurred. This does not affect our identification since the difference between the two isoforms involves residues 15–19 (Leszyk et al., 1987).

Labeling of Cardiac TnI. Rabbit cardiac TnI was labeled with iodo[^{14}C]acetamide both individually in Mg $^{2+}$ buffer and in the native RC-Tn complex in EDTA, Mg $^{2+}$, and Ca $^{2+}$ buffers. The two Cys residues, 75 and 92, of cardiac TnI were stoichiometrically labeled (82–94%) when the individual subunit was reacted with iodoacetamide (Table II). In contrast, when either native cardiac Tn complex was labeled, the apparent reactivity of the two Cys residues of TnI dropped dramatically (7–8%, Table II). To determine which subunit, TnT or TnC, has this effect on TnI, we used iodo[^{14}C]acetamide to label the TnCI and TnIT binary complexes. As shown in Table II, the Cys residues are stoichiometrically labeled (82–86%) in the TnCI complex and hence must be exposed on the surface of the subunit in this complex. In contrast, the extent of labeling of TnI in the TnIT complex is reduced by 48% to 65% when TnI is labeled in the presence of a 1.3-fold or 4-fold molar excess, respectively, of TnT. Due to the solubility problems encountered with the bovine cardiac TnIT complex (Hincke et al., 1979), labeling was performed at a higher ionic strength (0.5 M NaCl rather than 0.3 M KCl) than was employed for the other experiments. It is possible that the partial TnI labeling observed in these TnIT experiments is due to incomplete complex formation. To examine this possibility, the sample containing a 4-fold excess of TnT was run on a Superose 12 FPLC gel filtration column. As

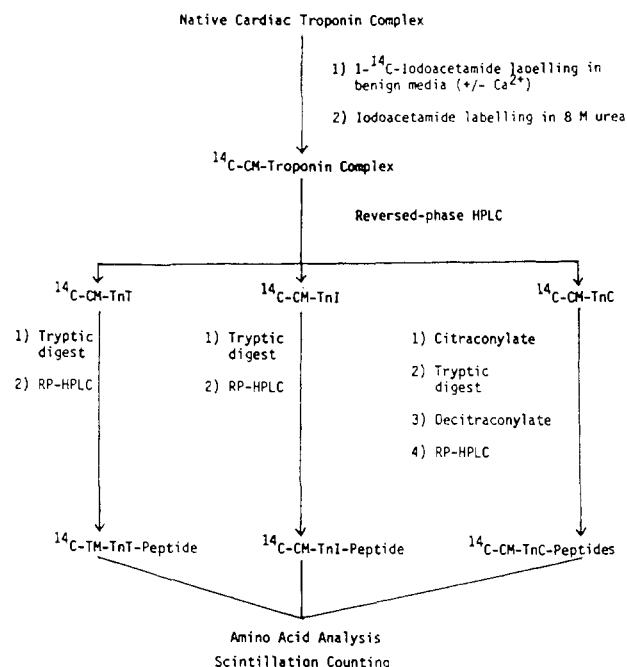


FIGURE 2: Scheme for the purification and analysis of the sulfhydryl groups of native Tn accessible to S-carboxamidomethylation. For details of the various steps, see Materials and Methods. Gradient conditions are described under Materials and Methods.

would be expected for incomplete complex formation, a peak corresponding to TnI eluted following the TnIT complex peak. These results suggest that Cys-75 and -92 of cardiac TnI are buried in the TnI-TnT interface in native Tn.

The results obtained for the cardiac TnT subunits demonstrate the necessity for determining whether any nonspecific labeling of residues other than Cys occurs when cardiac TnI reacts with iodoacetamide under our conditions. If this is not done, a degree of ambiguity in the interpretation of the data must result. For example, the apparent degree of modification of Cys-75 and -92 is 7–8% for TnI labeled within native Tn. This could imply that the TnI-TnT interaction which largely prevents these residues from reacting with the probe allows for a significant degree of “breathing” so that one or both residues are intermittently exposed during the course of the reaction. Alternatively, the 7–8% apparent labeling observed may be an artifact due to nonspecific labeling. Tryptic digestions of labeled cardiac TnI were performed and the appropriate fragments purified in order to address this issue. The RP-HPLC elution profile from the digests of RC-TnI yields two fragments (Figure 3) with amino acid compositions corresponding to the tryptic fragment containing residues 75–93 (data not shown). The two radioactive fragments do not differ in their respective levels of Cys modification. Sequencing of the two fragments from RC-TnI revealed that the first fragment was identical with the corresponding sequence in RC-TnI published by Grand et al. (1976), whereas in the second fragment, Gln-76 is replaced by a Leu residue. Consequently, it is clear that rabbit cardiac TnI consists of two isoforms. This result is consistent with the finding of two BC-TnI isoforms by Cachia et al. (1985), who utilized two-dimensional gel electrophoresis and denaturing ion-exchange chromatography in their study.

Significantly, the percentage labeling of Cys-75 and -92 in the tryptic fragments from the two isoforms is less than 1% when native Tn is modified under the various buffer conditions (Table II). This suggests, first, that the TnI-TnT interaction in this region is sufficiently strong to preclude any “floppiness” in the interaction which would allow partial exposure of Cys-75

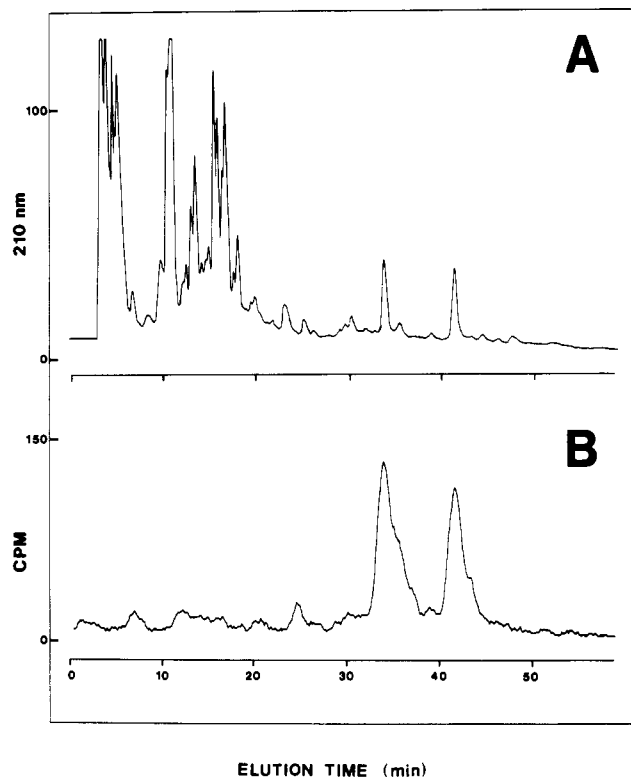


FIGURE 3: Separation of RC-TnI tryptic fragments from TnI labeled alone with iodo[^{14}C]acetamide in EDTA buffer on an analytical C_{18} RP-HPLC column. Panel A is the elution profile monitored at 210 nm. Panel B was obtained from continuous flow monitoring of radioactivity. Flow rate was 1 mL min^{-1} , and gradient conditions are described under Materials and Methods.

and -92 and, second, that the nonconservative replacement in residue 76 of a Leu for a Gln does not disrupt the interaction.

TnC Results. Labeling of the Cys residues of cardiac TnC ($\pm \text{Ca}^{2+}$) provides insight into the nature of the Ca^{2+} -induced conformational changes which the subunit undergoes in initiating contraction. Cardiac TnC contains two Cys residues, Cys-35 and Cys-84, both of which are located in the N-terminal domain of TnC where the contractile activation signal originates. In order to evaluate how the two Cys residues are affected by Ca^{2+} binding, both native BC-Tn and BC-TnC were labeled in the presence and absence of Ca^{2+} . The TnC obtained from these reactions was then citraconylated with citraconic anhydride (to limit cleavage to Arg residues) and subjected to tryptic digestion. Following this, the citraconate group was removed from the Lys residues of the protein fragments by exposure to acidic conditions, and the various fragments were isolated by RP-HPLC. Figure 4 contains elution profiles for the fragments obtained from BC-TnC labeled individually, plus and minus Ca^{2+} . The order of elution of the fragments is Cit 3 (84-102), Cit 5 (148-161), Cit 4 (103-147), and Cit 1 (1-46). The remaining Cit 2 (47-83) fragment was not isolated and is assumed by us to be the primary component of the precipitate which forms during the acidic decitraconylation step. This assumption is supported by the observation of Collins et al. (1977) that the related CNBr fragment CB8 (45-78) from rabbit skeletal TnC is insoluble under mildly acidic conditions. Surprisingly, the Cit 3 fragment (84-102) which comprises most of the D/E interdomain helical linker always eluted as two peaks (3a and 3b) probably due to incomplete decitraconylation. Both peaks were always radiolabeled to the same extent and appeared to have identical amino acid compositions. Sequencing of the two fragments demonstrated that the fragments were identical

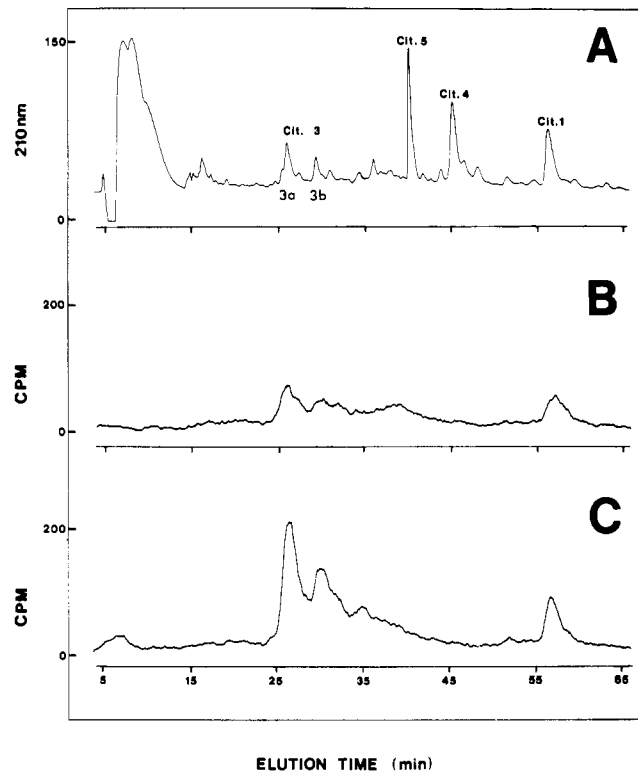


FIGURE 4: Separation of BC-TnC tryptic fragments from citraconylated TnC from Tn labeled with iodo[^{14}C]acetamide on an analytical C_8 RP-HPLC column. Panel A is the elution profile monitored at 210 nm. Panels B and C were obtained from continuous flow monitoring of radioactivity and correspond to labeling in the absence and presence of Ca^{2+} , respectively. Flow rate was 0.5 mL min^{-1} , and gradient conditions are described under Materials and Methods.

Table III: Percent Labeling of Cys Residues in Cardiac TnC with iodo[^{14}C]acetamide

sample	labeling conditions	
	EDTA (%)	Ca^{2+} (%)
BC-TnC		
Cit 1 (1-46)	11	16
Cit 3 (84-102)	26	63
BC-TnC _{Tn} ^a		
Cit 1 (1-46)	20	20
Cit 3 (84-102)	52	78

^a BC-TnC_{Tn} indicates subunit labeling within the Tn complex.

in sequence. However, for fragment 3b, both PTH-Lys and a peak corresponding to PTH-Tyr were observed at the position where Lys was expected. Since Tyr was not present in the amino acid analysis of the sample, it is possible that the apparent Tyr peak corresponded to Lys which had not been fully decitraconylated. Such a species would contain one rather than two PTH moieties, since the $\epsilon\text{-NH}_2$ group would be blocked. Interestingly, during their sequencing of BC-TnC, van Eerd and Takahashi (1976) purified four peaks corresponding to Cit 3 from a DEAE-Sephadex A-25 column. None of their other tryptic fragments from citraconylated TnC exhibited this behavior, which they also attributed to incomplete decitraconylation.

The percent labeling values of the fragments containing the two Cys residues are displayed in Table III. For TnC labeled alone, the Cys-35 residue located in the Cit 1 fragment was only partially labeled, 11% and 16% in the absence and presence of Ca^{2+} , respectively. For TnC reacted with iodo[^{14}C]acetamide in the native Tn complex, Cys-35 was 20% labeled regardless of the presence or absence of Ca^{2+} . Hence, for either the individual subunit or the native complex, Cys-35

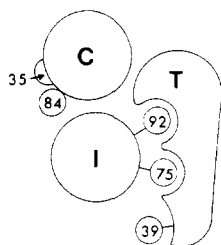


FIGURE 5: Schematic representation of the BC-Tn complex in the presence of Ca^{2+} showing the exposure of Cys sulfhydryl groups to reaction with iodoacetamide. The symbolism for the proteins is as follows: C, troponin C; I, troponin I; T, troponin T. The numbers represent the positions of the Cys residues in the various subunits.

is only marginally reactive. Cysteine-84, located in the Cit 3 fragment, was labeled 26% ($-\text{Ca}^{2+}$) and 63% ($+\text{Ca}^{2+}$) when TnC was labeled individually, and 52% ($-\text{Ca}^{2+}$) and 78% ($+\text{Ca}^{2+}$) when the native Tn was used. These results suggest two important conclusions. First, the presence of Ca^{2+} produces a significant increase in the apparent accessibility of the Cys-84 residue both in the individually labeled subunit and in the native complex. Second, the conformation of TnC in the Tn complex is such that Cys-84 is more reactive than it is for the individual subunit. This suggests that the region of TnC containing Cys-84 is not hidden in a subunit binding interface and that TnI and/or TnT imposes conformational constraints on TnC which alter the reactivity of Cys-84 (Table III).

DISCUSSION

The accessibilities of the Cys residues of cardiac Tn to reaction with iodoacetamide in native Tn, in the presence of Ca^{2+} , are schematically summarized in Figure 5. Protection of Cys residues from reaction with iodoacetamide can occur as a result of three different situations: first, the $-\text{SH}$ group is protected, buried within its subunit regardless of the other members of the complex; second, steric blocking in which the $-\text{SH}$ groups are located in a subunit interface; third, conformationally induced protection of the $-\text{SH}$ group within its own subunit as a consequence of binding Ca^{2+} or another subunit at a site distant from the Cys residues. When possible, these three situations will be distinguished.

The single Cys residue in the two isoforms of BC-TnT was completely accessible for reaction with iodoacetamide in native Tn ($\pm\text{Ca}^{2+}$), in TnT labeled alone, in BC-TnIT, and in the Tn-TM complex. All but the last of these results were expected for two reasons. First, the NH_2 -terminal segment of RC-TnT and the BC-TnT isoforms (containing either Cys-34 or Cys-39) is highly charged, with 26 of the first 50 residues being acidic (Pearlstone et al., 1986; Leszyk et al., 1987). Hence, this region could be expected to interact primarily with solvent rather than folding into a compact globular structure. To evaluate the probability that this Cys residue is indeed exposed to solvent, we have used a microcomputer program written in our laboratory (Parker et al., 1986) which uses hydrophilicity, accessibility, and mobility parameters for the various amino acid residues to predict surface regions in a protein sequence. In the surface profile for BC-TnT-1 (data not shown), virtually all of the residues between residues 10 and 56 are predicted to be on the surface including Cys-39. This observation explains why the Cys residues of individual BC-TnT molecules are expected to react with iodoacetamide under benign conditions. Our finding that Cys-39 was accessible in the various protein complexes studied agrees with our understanding of the topography of the Tn and Tn-TM complexes. The TnT molecule is well-known to be highly

asymmetric and to span an extensive region of the COOH-terminal third of TM in its interaction with that molecule (Ohtsuki, 1979; Mak & Smillie, 1981; Byers & Kay, 1983; Flicker et al., 1982). Data from several laboratories indicate that the NH_2 -terminal region of TnT is fairly distant (13 nm) from the TnT COOH-terminal region which interacts with TnI and TnC (Ohtsuki, 1979; Leavis et al., 1986). Consequently, it is improbable that their interaction with TnT would protect Cys-39 located in the NH_2 -terminal region from iodoacetamide.

The possibility that Cys-39 would be protected from labeling in the Tn-TM complex was anticipated to be somewhat greater than in the Tn complex alone, since the role of the NH_2 -terminal region of TnT from rabbit skeletal muscle is thought to involve binding to and stabilizing the head to tail junctions of contiguous TM molecules (Brisson et al., 1986; Pearlstone et al., 1986). Tobacman and Lee (1987) compared the abilities of BC-Tn containing the two BC-TnT isoforms to regulate the acto-S1-TM ATPase and have demonstrated a slight difference in the Ca^{2+} sensitivities of Tn containing the different isoforms. Since the difference between these isoforms in the NH_2 -terminal region of the molecule is simply the insertion of five residues, residues 15–19 of TnT-1, it appears that this portion of the NH_2 terminus is involved in regulation. Because our data indicate that Cys-39 is exposed in the cardiac Tn-TM complex, the question of whether or not this portion of the NH_2 -terminal region of cardiac TnT interacts with TM is left unanswered.

In the case of cardiac TnI, we have observed that Cys residues 75 and 92 are exposed when the protein is labeled either alone or in the TnCI complex but protected from iodoacetamide labeling in native Tn or in the TnIT complex. These observations suggest that the region of TnI in which these residues are located is either buried in a TnI-TnT binding interface or becomes protected as a result of conformational change when TnT binds to TnI. This conclusion is not surprising in light of earlier observations that the BC-TnI-TnT interaction is significantly weakened by carboxamidomethylation of the two Cys residues of BC-TnI (Hincke et al., 1979) and that rabbit skeletal TnI and TnT do not bind each other if an intramolecular disulfide bond forms between Cys-48 and -64 of RS-TnI (Horwitz et al., 1979). In addition, other data obtained in studies of RS-Tn subunit interactions are consistent with our findings for the cardiac Tn system. Cysteines-75 and -92 of cardiac TnI are conserved as Cys-48 and -64 in RS-TnI, and as in the case of skeletal TnI, both residues are exposed when either TnI or TnCI (minus Ca^{2+}) is labeled but are inaccessible to modification when either native Tn or TnIT is labeled (Chong & Hodges, 1982a). Similarly, Hitchcock-DeGregori (1982) found that the Lys residues in RS-TnI corresponding to Lys-67, Arg-93, and Arg-106 in RC-TnI are significantly less reactive with [^3H]-acetic anhydride in the RS-TnIT complex than in RS-TnI alone. Having determined that TnT causes the TnI Cys residues to become inaccessible to modification, the following question arises: Is this due to burial of the region of TnI containing these residues in a subunit interface or to a conformational change resulting from complex formation? Our experimental approach in this study is incapable of resolving this question. However, it is quite probable that the former explanation is the correct one. Chong and Hodges (1982b) used the heterobifunctional photoaffinity cross-linking reagent AGTC to investigate the interaction between RS-TnI and the other RS-Tn subunits in native Tn. Upon activation, AGTC probes attached to Cys-48 and Cys-64 of RS-TnI reacted to

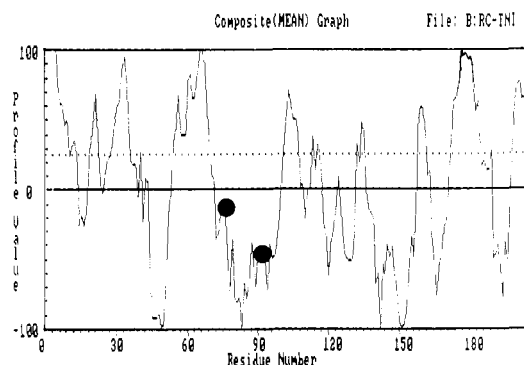


FIGURE 6: Surface profile for RC-TnI calculated from the sequence using hydrophilicity, accessibility, and mobility parameters described by Parker et al. (1986). The solid line represents the mean protein profile value and the dotted line the 25% cutoff value. Residues above this line are predicted to be surface sites.

yield 35% intramolecular cross-linking, 55% cross-linking to TnT and only 10% cross-linking to TnC. These results demonstrate that a binding site for TnT is located in this region of TnI. Consequently, formation of the TnIT complex could be expected to render the TnI Cys residues inaccessible to iodoacetamide. Further insight into this matter is gained from the surface prediction plot for RC-TnI shown in Figure 6. It is apparent from this plot that the region of TnI containing Cys-75 and -92 is relatively hydrophobic and is predicted not to be exposed on the protein surface. The fact that it is exposed only in the absence of TnT strongly suggests that it functions as a hydrophobic binding interface for TnT and that protection of the Cys residues is due to this rather than due to TnT-induced conformational change.

Although the crystal structures of both turkey skeletal TnC (Herzberg & James, 1985) and chicken skeletal TnC (Sundaralingham et al., 1985; Satyshur et al., 1988) have been solved, the precise nature of the changes which occur in TnC upon Ca^{2+} binding to the N-terminal domain is unknown. Herzberg et al. (1986) have attempted to answer this question by using the crystal structure of turkey skeletal TnC, computer modeling, and energy minimization. The crystal structure of turkey TnC contains Ca^{2+} in the two Ca^{2+} - Mg^{2+} sites of the C-terminal domain but empty Ca^{2+} -specific sites in the N-terminal domain. This structure is thought by Herzberg et al. (1986) to model the structure of TnC in the relaxed state. On the basis of the assumption that the structure of the N-terminal domain with Ca^{2+} bound to it would closely resemble that of the C-terminal domain crystal structure, these authors generated a model of the Ca^{2+} -activated state. According to the crystal structure of turkey skeletal TnC, Gln-85, the residue corresponding in sequence position to Cys-84 of BC-TnC, is quite buried with a solvent accessibility of only 0.1 \AA^2 in the absence of Ca^{2+} . In contrast, the solvent accessibility of this residue in the Ca^{2+} -activated model is 50.9 \AA^2 due to a large shift in the positions of residues from the B and C helices and the peptide linker connecting these helices. The increase in reactivity of Cys-84 when Ca^{2+} is present is in qualitative agreement with the prediction of a large increase in solvent accessibility by Herzberg et al. (1986) for Gln-85 and provides experimental support for their Ca^{2+} -activated state model. Furthermore, our observation that Cys-84 is more exposed in the Tn complex than in TnC alone, both in the presence and in the absence of Ca^{2+} , suggests that TnI and/or TnT imposes constraints on TnC which to some degree shift its structure toward that of the Ca^{2+} -activated structure. This interpretation is reasonable since several laboratories have shown that Ca^{2+} and TnI binding to skeletal TnC is coupled by a negative

interaction free energy (Potter & Gergely, 1975; Ingraham & Swenson, 1984; Cheung et al., 1987). This type of coupling occurs when two ligands stabilize similar conformations of the protein molecule that they both bind to (Weber, 1972).

One notable difference between our results and those of Herzberg et al. (1986) concerns the accessibility of Gln-85 (corresponding to Cys-84) in the relaxed state. On the basis of the crystal structure, this residue would not be expected to undergo any significant degree of labeling in the minus Ca^{2+} state. Our observation of partial labeling may arise from the higher pH used in our study (pH 7.5) compared to pH 5.0 for the crystallographic investigation. This explanation is supported by the fact that the carboxyl-carboxylate interaction between Glu-88 and Glu-57 of helix C observed in the turkey TnC crystal structure would not exist at pH 7.5. This is significant because this interaction helps restrict access to Gln-85 (Herzberg, private communication). Alternatively, "breathing motions" of the polypeptide backbone may be responsible, or both mechanisms may be involved.

As discussed previously, Cys residues can be quite useful as points of attachment for a wide variety of structural probes. In light of our knowledge of the accessibility of the Cys residues of cardiac Tn, the following conclusions can be made about their suitability for probe attachment. Cysteine-39 of BC-TnT is completely exposed both in the native complex and in the Tn-TM regulatory complex. Since it is not part of a protein interface, it is unlikely that a probe attached to it will interfere significantly with the interaction or phenomenon to be studied. Given its location in the NH_2 -terminal region, structural probes attached to Cys-39 could be very useful in examining the interaction between this region and the head to tail overlap region of TM. Cysteines-75 and -92 from cardiac TnI are both buried in the TnI-TnT interface and exposed in the TnCI complex. Consequently, labeling of cardiac TnI will necessarily involve both Cys residues. Significantly, the fact that probes bound to these residues are in an interface in Tn does not preclude their utility in studying the TnI-TnT interaction as Chong and Hodges (1982b) have shown in their cross-linking study using the skeletal Tn system. In the case of the TnC, the two Cys residues, 35 and 84, exhibit differential labeling in that Cys-84 is more reactive than Cys-35. Factors which enhance the reactivity and selectivity for labeling of Cys-84 are the presence of Ca^{2+} and being within the Tn complex. However, under all conditions examined, a significant degree of partial labeling of Cys-35 also occurs (11–20%). For many experiments, the heterogeneity in labeled molecules introduced as a consequence should not be a major problem, and studies utilizing cardiac TnC and cardiac Tn should complement those already obtained using the skeletal Tn system.

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