Covalent Binding of Peptides to the N-terminal Hydrophobic Region of Cardiac Troponin C Has Limited Effects on Function*

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Xin Lin†, Darrell G. Dotson, and John A. Putkey§
From the Department of Biochemistry & Molecular Biology, The University of Texas Medical School, Houston, Texas 77030

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†Current address: Gladstone Institute of Virology and Immunology, University of California, San Francisco, P. O. Box 419100, San Francisco, CA 94141-9100.
§Recipient of a Research Career Development Award from the National Institutes of Health. To whom correspondence should be addressed: Dept. of Biochemistry & Molecular Biology, the University of Texas Medical School, 6431 Fannin St., Houston, TX 77030. Tel.: 713-792-5604; Fax: 713-794-4150; E-mail: jputkey@utmmg.med.uth.tmc.edu.

Covalent Binding of Peptides to the N-terminal Hydrophobic Region of tropolin C is thought to be important for the regulation of contraction in striated muscle. To test this hypothesis, single Cys residues were engineered at positions 45, 81, 84, or 85 in the N-terminal hydrophobic region of cardiac tropolin C (cTnC) to provide specific sites for attachment of blocking groups. A synthetic peptide, Ac-Val-Arg-Ala-Ile-Gly-Lys-Leu-Ser-Ser, or biotin was coupled to these Cys residues, and the covalent adducts were tested for activity in TnC-extracted myofibrils. Covalent modification of cTnC(C45) had no effect on maximal myofibrill ATPase activity. Greatly decreased myofibrill ATPase activity (70–80% inhibited) resulted when the peptide was conjugated to Cys-81 in cTnC(CB1), while a lesser degree of inhibition (10–25% inhibited) resulted from covalent modification of cTnC(C84) and cTnC(C85). Inhibition was not due to an altered affinity of the cTnC(C81)/peptide conjugates for actin-myosin, and the Ca2⁺ dependence of ATPase activity was essentially identical to the unmodified protein. Thus, a subregion of the N-terminal hydrophobic region in cTnC is sensitive to disruption, while other regions are less important or can adapt to rather bulky blocking groups. The data suggest that Ca2⁺-sensitizing drugs may bind to the N-terminal hydrophobic region on cTnC but not interfere with transmission of the Ca2⁺ signal.

Regulation of contraction and relaxation in vertebrate striated muscle relies on the binding and release of Ca²⁺ from troponin C (TnC) in the troponin complex. Kinetics studies (1, 2) and site-directed mutagenesis (3, 4) have demonstrated that the N-terminal Ca²⁺ binding sites I and II in skeletal TnC (sTnC) and site II in cardiac TnC (cTnC) are primarily responsible for this regulation. Binding of Ca²⁺ or Mg²⁺ to the C-terminal high affinity sites in TnC facilitates tight association of TnC with thin filaments in the relaxed state (5–7).

Although the molecular mechanism by which TnC regulates muscle contraction has not been rigorously defined, it is thought to involve two steps. The first step is based on the molecular modeling studies of Herzberg et al. (6), which predicted that helices B and C of TnC move away from helices A and D upon Ca²⁺ binding to the low affinity sites, thereby exposing a hydrophobic surface. This step of the model has found experimental support (9–11) and has recently been confirmed by resolution of the solution structures for apo- and Ca²⁺-bound sTnC (12). In the second step of the model, the exposed N-terminal hydrophobic region in TnC is thought to associate with a complimentary region in TnI, thereby releasing inhibition of actomyosin ATPase. This is an attractive concept, since it is analogous to the interaction of calmodulin (CaM) with a subset of target proteins.

If an exposed hydrophobic region participates in the second step of the model, and is important for transmission of the Ca²⁺ signal, then it is logical to predict that regulation of contraction would be disrupted by ligands which bind to and block the hydrophobic surface. A number of small aromatic drugs which bind to and inhibit the activity of CaM or TnC have been shown to inhibit cardiac muscle contraction (16–18). However, these compounds sensitize, rather than inhibit, cardiac muscle contraction (16–18). Thus, the role of the N-terminal hydrophobic region in the mechanism of action of sTnC or cTnC is not clear.

Site-specific mutation of multiple hydrophobic amino acids in the N-terminal domain of cTnC could potentially test the importance of this region, but significant artifacts could result from abnormal protein folding. To overcome these problems, we chose to covalently attach bulky groups to specific sites in the N-terminal hydrophobic region of cTnC. We then compared the functional properties of the protein conjugates with the unmodified proteins. Attachment of a synthetic peptide or biotin to single Cys residues placed at position 45, 84, or 85 had little or no functional consequence. In contrast, the activity of cTnC was greatly inhibited by coupling of these blocking groups to Cys at position 81. Thus only limited portions of the N-terminal hydrophobic region appear to be important for transmission of the Ca²⁺ signal.

MATERIALS AND METHODS

Mutant Proteins—Wild-type recombinant cTnC3, cTnC(C84), and cTnC(A-Cys) were generated as described previously (3, 19). Expression plasmids encoding cTnC proteins with single Cys residues at positions 42, 45, 81, or 85 were constructed by the method of polymerase chain reaction splicing by overlap extension (20) using a plasmid encoding cTnC(A-Cys) as a template. Sequence fidelity and mutations were confirmed by dideoxy-DNA sequencing. Mutant proteins were expressed and purified to homogeneity as described previously (21).

Preparation of cTnC-Peptide Conjugates—The synthetic peptide, Ac-Val-Arg-Ala-Ile-Gly-Lys-Leu-Ser-Ser, or biotin was coupled to these Cys residues, and the covalent adducts were tested for activity in TnC-extracted myofibrils. Covalent modification of cTnC(C45) had no effect on maximal myofibrill ATPase activity. Greatly decreased myofibrill ATPase activity (70–80% inhibited) resulted when the peptide was conjugated to Cys-81 in cTnC(CB1), while a lesser degree of inhibition (10–25% inhibited) resulted from covalent modification of cTnC(C84) and cTnC(C85). Inhibition was not due to an altered affinity of the cTnC(C81)/peptide conjugates for actin-myosin, and the Ca²⁺ dependence of ATPase activity was essentially identical to the unmodified protein. Thus, a subregion of the N-terminal hydrophobic region in cTnC is sensitive to disruption, while other regions are less important or can adapt to rather bulky blocking groups. The data suggest that Ca²⁺-sensitizing drugs may bind to the N-terminal hydrophobic region on cTnC but not interfere with transmission of the Ca²⁺ signal.
Val-Ala-Ile-Gly-Lys-Leu-Ser-Ser, was purified by reverse phase HPLC, lyophilized, and reacted with the heterobifunctional cross-linking reagent succinimidyl-3-(2-pyridyldithio)propiononate (SPDP) (Molecular Probes) in 0.1 M NaHCO₃, pH 8.5, at an SPDP:peptide molar ratio of 2:1. After reacting for 30 min, the SPDP-activated peptide was re-purified by reverse phase HPLC. Prior to attaching the activated peptide to the proteins, the cTnC monocysteine derivatives were reduced in 20 mM dithiothreitol (DTT) at 37 °C for 1 h and desalted into 0.1 M NaHCO₃ using Bio-Rad P6DG gel filtration resin. The reduced cTnC monocysteine proteins were then mixed with the SPDP-activated peptide in 0.1 M NaHCO₃, pH 8.5, at a peptide:protein molar ratio of 2:1. cTnC(C42), cTnC(C84), and cTnC(C85) were labeled in the presence of 1 mM CaCl₂, whereas cTnC(C45) and cTnC(C81) were labeled in 1 mM EGTA. The absorbance of each labeling reaction was monitored at 343 nm, corresponding to the peak absorbance of the released chromophore, 2-pyridinedithione. All cTnC-peptide conjugates were concentrated by ultrafiltration (Centricon, Amicon Co.) and desalted into 50 mM MOPS, pH 7.0, using Bio-Rad P6DG gel filtration resin to remove unreacted reagents.

**Blocking of the N-terminal Hydrophobic Region in cTnC**

Myofibril ATPase Assays—TnC-depleted cardiac and skeletal myofibrils were prepared and ATPase assays were performed as described previously (7). In some experiments, the peptide-TnC conjugates were incubated for 1 h at 4 °C either with or without 8 mM DTT, prior to addition to the TnC-depleted myofibrils. The Ca²⁺ ATPase activity at a concentration of 0.1 mM and has no effect on the fluorescence signal from cTnC(C81) labeled with none-4-maleimide (BP-Mal) as described previously for sTnC by Tao et al. (22). First, cTnC(C81) was reduced with 10 mM DTT for 1 h at 37 °C and dialyzed overnight against 20 mM HEPES, pH 7.5, 0.1 mM NaCl, and 0.1 mM DTT. BP-Mal was reacted with the reduced cTnC(C81) for 2 h at room temperature at a BP-Mal:protein molar ratio of 1.5:2.5:1. The reaction was quenched with excess DTT and dialyzed against 50 mM MOPS, pH 7.0, to remove excess reagents. cTnC(A-Cys) was used as a negative control to demonstrate the extent of nonspecific labeling. The degree of labeling was determined by nondenaturing PAGE in the presence and absence of avidin.

**RESULTS**

**cTnC Monocysteine Mutants and Blocking Groups**—Fig. 1A lists the recombinant proteins used in the current study, and Fig. 1B shows the relative positions of sulfur atoms of the Cys residues in the monocysteine proteins. Cys-84 is one of the two endogenous Cys residues of cTnC. Residue 45, 81, and 85 are Met residues in the wild-type protein and are predicted to form part of the N-terminal hydrophobic surface of cTnC. Residues 45 and 81 are of particular interest, since our previous studies showed that the C¹³ and CH³ chemical shifts of Met-45 and Met-81 change significantly upon Ca²⁺ binding to the low affinity site I (21). Gly-42 was converted to Cys as a negative control, since it is predicted to be on the solvent accessible surface of helix B, based on the crystal structure of sTnC (23) and a model of cTnC (24).

**Table A**

| Protein          | Mutation |
|------------------|----------|
| cTnC(A-cys)      | C38S, C84S |
| cTnC(C42)       | G40C, C35S, C84S |
| cTnC(C45)       | M46S, C35S, C84S |

**Table B**

| Protein          | Mutation |
|------------------|----------|
| cTnC(C81)       | M81C, C38S, C84S |
| cTnC(C84)       | C38S |
| cTnC(C85)       | C38S, M85C, C84S |

**Fig. 1. Location of Cys residues and nomenclature for monocysteine mutants.** A lists the nomenclature and specific mutations in the cTnC proteins used in this study. B shows a space filling model of the peptide backbone of amino acids 1-95 from the Ca²⁺-bound form of cTnC as well as the side chains of the Cys residues. The relative positions of the sulfur atoms are indicated in black. This structure was generated using a model of cTnC reported previously (24). The space filling model on the right represents the peptide backbone of a nine amino acid peptide in an α-helical conformation.

A commercially available sulfhydryl-specific maleimide derivative of biotin was selected as one blocking group. Peptides were also considered as blocking groups, since they can be synthesized with specified lengths and chemical properties for desired solubility and coupling. Several criteria were considered in selection of the peptide sequence. Sufficient size was necessary to prevent an effective blocking group but not so large as to interact, either specifically or nonspecifically, with the C-terminal half of cTnC and thus interfere with binding to the thin filament. It is not necessary that the peptide have an intrinsic affinity for cTnC, but we felt that it should be designed based on available data for the interaction of peptides with either TnC or CaM rather than a more arbitrary sequence. Finally, the point of coupling should be near the middle of the peptide to prevent it from being displaced from its intended position by rotating about its end point.

Given these criteria, we chose a peptide (Ac-Val-Arg-Ala-Ile-Gly-Lys-Leu-Ser-Ser) based on amino acids 807–815 from smooth muscle myosin light chain kinase which binds to the N-terminal half of CalM (25, 26). The N-terminal Val was acetylated to provide a reactive amine on the Lys. Fig. 1B shows the relative size of the backbone of cTnC and the peptide. The peptide is depicted in an α-helix which would represent the minimal effective volume. A derivative of the minimal inhibitory peptide of cTnC or sTnC was not considered, since it preferentially associates with the C-terminal domain of intact sTnC and cTnC (27–32). Such an association might result in nonspecific interactions in cTnC, but we felt that it should be designed based on available data for the interaction of peptides with either TnC or CaM rather than a more arbitrary sequence. Finally, the point of coupling should be near the middle of the peptide to prevent it from being displaced from its intended position by rotating about its end point.

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doacetyl-N’-(5-sulfo-1-naphthyl)ethylenediamine (data not shown).

Covalent Modification of cTnC—Fig. 2A shows the strategy used to cross-link the peptide to cTnC with the heterobifunctional cross-linker SPDP. The release of 2-pyridinethione was monitored spectrophotometrically to determine the extent of coupling. Fig. 2B shows that 10 mM DTT is required to completely reduce the disulfide bond between the peptide and cTnC(C81). Similar results were obtained with the other cTnC proteins. Thus, coupling of the peptide to the monocysteine proteins was essentially complete and cTnC-Pep adducts were stable but reversible.

Biotin was coupled to cTnC by irreversible alkylation of Cys residues at pH 7.5. cTnC(A-Cys), which does not contain Cys residues, was used as a negative control, since maleimide can react with amines at a pH greater than 8.0. Native gels run in the presence or absence of avidin were used to determine the extent of coupling. Fig. 3 shows that about 90% of each monocysteine protein was biotinylated, while only a trace amount of cTnC(A-Cys) was biotinylated.

Activity of Monocysteine Derivatives and Peptide Adducts—Fig. 4A shows that all unmodified cTnC proteins recovered similar levels of Ca\(^{2+}\)-dependent ATPase activity in cTnC-extracted cardiac myofibrils. Fig. 4B shows that the monocysteine derivatives exhibited greater variability in their ability to recover activity in sTnC-extracted fast skeletal myofibrils, but that all recovered at least 70% of the activity recovered by cTnC3.

Table I compares the activities of the cross-linked proteins relative to the corresponding unmodified proteins. Covalent coupling of the peptide to Cys-81 resulted in 70–80% inhibition of cTnC activity in cardiac and skeletal myofibrils, respectively. The residual activity was not due to a significant amount of unmodified protein, since greater than 95% of cTnC(C81) was coupled to the peptide. The decreased Ca\(^{2+}\)-dependent activity of cTnC(C81)-Pep results from a 10% increase in Ca\(^{2+}\)-independent activity and a 60–70% decrease in total activity. Although residues 45, 84, and 85 are all predicted to form the N-terminal hydrophobic surface, coupling the peptide to these residues had resulted in little or no decrease in activity. Coupling the peptide to residue 42 resulted in a slight increase in activity.

Activity of cTnC-Biotin Adducts—The activities of cTnC-biotin adducts were determined using the skeletal myofibril ATPase assay. Table II shows the activities of the cTnC-biotin adducts in the presence and absence of Ca\(^{2+}\). The values are
expressed as a percent of the maximal activity observed for the corresponding unmodified protein in the presence of Ca\(^{2+}\). The activity of cTnC(C45)-biotin in the presence of Ca\(^{2+}\) was not significantly different from the unmodified protein, but its Ca\(^{2+}\)-independent activity was significantly increased. Biotinylation of cTnC(C84) and cTnC(C85) caused modest decreases in activity. This is consistent with the slight decrease in activity seen for cTnC(C84)-Pep and cTnC(C85)-Pep in Table I. Similar to the results with the peptide conjugate, biotinylation greatly decreased the activity of cTnC(C81). These results confirm that the activity of cTnC is most affected by covalent modification of residue 81 in cTnC.

Preliminary experiments showed that binding avidin to cTnC(C42)-biotin had no effect on function, while avidin further decreased the activity of cTnC(C81)-biotin (data not shown). Additional experiments using avidin were not pursued, since labeling with biotin was not quantitative (see Fig. 3) and since the large size of avidin (60,000 Da) increases the possibility that observed functional consequences are due to disruption of interactive sites that are distal to the site of attachment.

Binding of cTnC(C81)-Pep to the Thin Filament and Its Ca\(^{2+}\) sensitivity—cTnC(C81)-Pep was selected for further characterization, since it has the most compromised activity. Fig. 5 shows that the Ca\(^{2+}\)-dependent activity of cTnC(C81)-Pep remains virtually unchanged over a 5-fold range of concentrations in both cardiac and skeletal myofibril ATPase assays. Other experiments showed that cTnC(C81)-Pep effectively competed with cTnC(A-Cys) for binding sites on the thin filament (data not shown). Thus, coupling the peptide to position 81 does not greatly alter the affinity of cTnC(C81) for the thin filament.

Conversion of nonpolar residues to polar residues in the N-terminal hydrophobic region of sTnC was shown to increase the Ca\(^{2+}\) affinity of the low affinity sites in the isolated sTnC (33) and to increase the pCa50 of skinned fiber contraction (34). Therefore, it was of interest to determine if covalent coupling of the peptide to cTnC(C81) affected the Ca\(^{2+}\) sensitivity of myofibril ATPase activity. Fast skeletal myofibrils were used for these experiments, since the specific activity of these preparations is about 10-fold greater than cardiac myofibrils. Fig. 6 shows that coupling the peptide to cTnC(C81) does not significantly alter the Ca\(^{2+}\) sensitivity of myofibril ATPase activity.

Cross-linking cTnC(C81) to cTnI—If the compromised activity of cTnC(C81)-Pep is due to a blocking effect, then the region of Cys-81 should be in close proximity to other troponin subunits. To test this possibility, cTnC(C81) was labeled with the heterobifunctional photo-cross-linker BP-Mal, which has been used to study interactions between sTnC and sTnl (30, 31, 35, 36). cTnC(A-Cys) was used as a control for cross-linking due to nonspecific modification of cTnC with BP-Mal. cTnC(C81)-BP or cTnC(A-Cys)-BP were cross-linked with either cTnl alone or with cTnl and cTnT. No significant cross-linking was observed using cTnC(A-Cys)-BP (data not shown). Fig. 7 shows that cTnC(C81)-BP effectively cross-links to cTnl when associated
The side chain of Met-45 is predicted to reside on the inner surface of helix B in the N-terminal hydrophobic region (see Fig. 1), and NMR studies (21, 28) indicate that the solvent accessibility of Met-45 increases upon binding Ca$^{2+}$. Never the less, covalent modification of residue 45 had little or no functional consequence. We cannot exclude the possibility that blocking groups attached to cTnC(C45) can rotate about the covalent bond, perhaps under the influence of cTnI, and assume a position which does not interfere with transmission of the Ca$^{2+}$ signal. If this occurs, it is likely that the bound peptide or biotin extends away from Met-81 (into the plane of the page in the model shown in Fig. 1). In any event, it is clear that localized structural alteration at residue 45 in the N-terminal hydrophobic region has little effect on the maximal activity of cTnC.

Covalent modification of cTnC(C81) with either the peptide or biotin resulted in significant inhibition of activity. Modification of cTnC(C84) and cTnC(C85) lowered activity relative to cTnC(C81) and cTnC(C82). Both studies concluded that these compounds establish hydrophobic interactions with residues in the N-terminal hydrophobic region of cTnC, including Met-81. Data predict that strong interaction of a drug with Met-81 would inhibit rather than enhance the function of cTnC. This apparent inconsistency could be explained by an incorrect assignment for the methyl proton chemical shift for Met-81 of 2.22 ppm used in the previous studies. We have assigned this chemical shift to 1.35 ppm based on site-directed mutagenesis and two-dimensional heteronuclear single and multiple quantum coherence NMR (21). Although we feel that it is likely that bepridil and levosimendan bind to the N-terminal domain of free cTnC, they may make primary interactions with Met residues other than Met-81.

If one assumes that the Ca$^{2+}$-sensitizing effect of anti-CaM drugs results from association with the N-terminal hydrophobic region of thin filament-bound cTnC, then at least three mechanisms that can be considered. First, the drugs may associate with a critical hydrophobic cTnC binding site and sensitize cTnC to Ca$^{2+}$, but then be displaced by cTnI when cTnC binds Ca$^{2+}$. Second, the N-terminal hydrophobic region of cTnC may be a critical Ca$^{2+}$-dependent site of interaction with cTnI, yet be able to simultaneously bind Ca$^{2+}$-sensitizing drugs or the covalently bound peptide. Third, the N-terminal hydrophobic region may be an essential Ca$^{2+}$-dependent binding site for cTnI, but can bind and sensitize cTnC to Ca$^{2+}$. Our data are generally consistent with the latter two mechanisms, since they show that discrete regions on the N-terminal hydrophobic surface can tolerate covalently bound groups, and presumably a noncovalently bound drug, without interfering with transmission of the Ca$^{2+}$ signal. This is also consistent with the study of Fasshauer et al. (33) which showed that conversion of selected nonpolar residues to polar residues in the N-terminal hydrophobic region of sTnC increased the Ca$^{2+}$ affinity of low affinity sites, but did not inhibit regulation. Thus, careful consideration must be given with respect to the precise role for the N-terminal hydrophobic region of cTnC in the regulation of cardiac muscle contraction.

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Fig. 7. SDS-PAGE of cTnC(C81)-BP cross-linked to cTnI or cTnT. Free cTnC(C81)-BP (lanes 2–4), cTnC(C81)-BP-cTnI binary complexes (lanes 5–7), and cTnC(C81)-BP-cTnI-cTnT ternary complexes (lanes 8–10) were prepared as described under "Materials and Methods." One aliquot of each preparation was maintained in the dark (lanes 2, 5, and 8), a second aliquot was irradiated with UV light in the presence of 0.1 mM Ca$^{2+}$ (lanes 3, 6, and 9), and a third aliquot was irradiated with UV light in the presence of 2 mM EGTA (lanes 4, 7, and 10). All samples were analyzed by SDS-PAGE. Lane 1, molecular mass standard.
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