Fluorescent Probes Attached to Cys 35 or Cys 84 in Cardiac Troponin C Are Differentially Sensitive to Ca^{2+}-Dependent Events *in Vitro* and *in Situ*†

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ABSTRACT: The goal of the current study was to generate recombinant cTnC proteins with single Cys residues as sites for attachment of fluorescent probes that can distinguish between the structural effects of myosin cross bridges and direct Ca^{2+} binding to cTnC (cardiac and slow skeletal troponin C) in skinned fibers. We anticipated that cTnC proteins which retain the endogenous Cys 35 (cTnC(C35)) or Cys 84 (cTnC(C84)) would provide fluorescent probes with distinct microenvironments, since these residues are on opposite sides of the globular regulatory domain. *In vitro* experiments that showed IAANS (2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid) coupled to Cys 35 can induce unwanted structural perturbations as evidenced by a decreased affinity of site II for Ca^{2+} when IAANS-labeled cTnC(C35) is bound to cTnI. Important structural features involving Cys 35 in the inactive site I are suggested by a Ca^{2+}-dependent increase in reactivity of Cys 35 with sulfhydryl specific reagents when cTnC(C35) is associated with cTnI. These characteristics are not seen for cTnC(C84). When incorporated *in situ* into skinned cardiac muscle fibers, native cTnC with IAANS bound to both Cys 35 and Cys 84 showed a pCa_{50} of fluorescence which preceded that of force, while the pCa_{50} values of both force and fluorescence were coincident for IAANS-labeled cTnC(C84). Disruption of force-producing myosin cross bridges had no effect on the pCa_{50} of fluorescence for IAANS-labeled cTnC(C84), but induced a rightward shift in the pCa_{50} of fluorescence for IAANS-labeled native cTnC. These data can be interpreted to indicate that cTnC with IAANS bound to both Cys 35 and C84 senses either myosin cross bridges or direct Ca^{2+} binding and myosin-induced cooperativity, while IAANS bound to Cys 84 alone senses conformations that are tightly coupled with force generation.

Contraction in striated muscle is dependent on the interaction of Ca^{2+} with skeletal troponin C (sTnC[1]) in fast skeletal muscle and cardiac troponin C (cTnC) in cardiac and slow skeletal muscle (for review see Gergely et al. (1993)). Normal regulation of muscle contraction is a complex process in which myosin cross bridges and cooperative interactions along the thin filament serve to modify the structure and the Ca^{2+}-binding affinity of TnC. An added level of complexity can be found in cardiac muscle in which variations in pH and phosphorylation of cardiac TnI have significant effects on Ca^{2+} binding to cTnC. Structural studies to resolve Ca^{2+}-dependent conformational changes of free TnC and its interactions with other troponin subunits can be accomplished using techniques such as NMR, X-ray crystallography, circular dichroism, etc. However, these techniques have limited application for the study of Ca^{2+}-dependent events involving TnC in functional myofibrils. For such studies, fluorescent probes which are covalently bound to TnC have provided a valuable tool to correlate structural changes in TnC with other calcium-dependent events in muscle fibers and thin filaments (Johnson et al., 1980; Putkey et al., 1989; Zot et al., 1986; Tobacman & Sawyer, 1990; Liao et al., 1994).

Fluorescence from DANZ-labeled sTnC incorporated into fast skeletal muscle fibers was activated at a lower Ca^{2+}

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[1] Abbreviations: TnC, both isoforms of troponin C; sTnC, fast skeletal troponin C; cTnC, cardiac and slow skeletal troponin C; cTnC3, bacterially synthesized cTnC(des M1, D2A); cTnC(C35), cTnC3(C84S); cTnC(C84), cTnC3(C35S); IAANS, 2-(4′-(iodoacetamido)anilino)-naphthalene-6-sulfonic acid; $cTnC_{IA}$, IAANS-labeled cTnC3; $cTnC(C35)_{IA}$, IAANS-labeled cTnC(C35); $cTnC(C84)_{IA}$, IAANS-labeled cTnC(C84); IAEDANS, 5-((2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; DANZ, dansylaziridine (((5-(dimethylamino)-naphthalen-1-yl)sulfonyl)aziridine); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CPM, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin.

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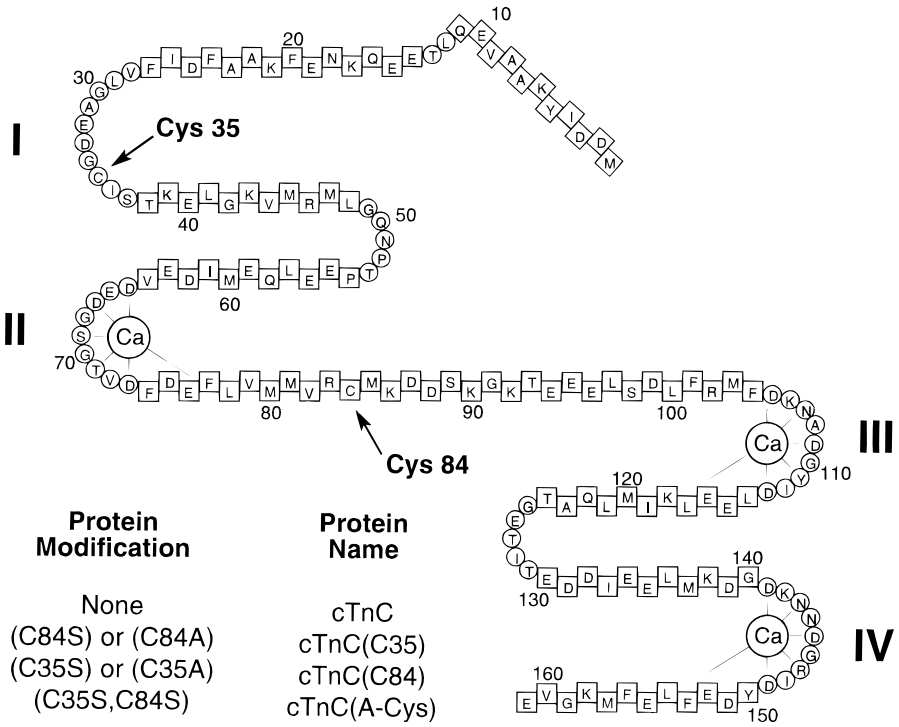

FIGURE 1: Summary of mutant proteins. The proteins used in this study are summarized with a diagrammatic representation of the primary structure and predicted secondary structure of chicken cTnC. The structure is based on the 3-dimensional structures for calmodulin and sTnC (Babu et al., 1985; Herzberg & James, 1985; Sundaralingam et al., 1985). Squares represent α-helical regions, and circles represent β sheets and random coils. Coordination bonds are represented by lines radiating from Ca^{2+}.

concentration than force, and it was concluded that fluorescence was affected by both rigor and cycling cross bridges (Zot et al., 1986; Guth & Potter, 1987). Kerrick et al. (1991), using skeletal fibers reconstituted with DANZ-labeled sTnC, showed that Ca^{2+}-dependent ATPase and fluorescence changes were more tightly coupled than were force and fluorescence, which suggests coupling between cross-bridge formation and fluorescence. Hannon et al. (1992) came to a similar conclusion to account for the observation that fluorescence from cTnC with IAANS coupled to Cys residues more closely parallels stiffness than force, and that both fluorescence and stiffness are activated at a lower Ca^{2+} concentration than force.

Although Cys residues allow quantitative and site-specific labeling of proteins, the two Cys residues in cTnC (amino acids 35 and 84) present both the possibility of unwanted structural perturbations and the possibility that the two probes may sense different Ca^{2+}-dependent events, i.e., cross bridges and/or direct Ca^{2+} binding. To eliminate this concern, we have generated monocys derivatives of cTnC which contain Cys 35 or Cys 84 in the N-terminal regulatory domain. These proteins and their covalent adducts were characterized *in vitro* to identify potential unwanted structural perturbations and then *in situ* in muscle fibers to correlate fluorescence changes with Ca^{2+} concentration and force generation. The data show that the Ca^{2+}-binding affinity of site II is affected by IAANS bound to Cys 35, but this is evident only when the IAANS-labeled protein is associated with cTnI. The data also indicate that the normal protein with two Cys residues and two covalently bound IAANS molecules appears to sense either cross-bridge attachment or direct Ca^{2+} binding and cooperative events, while fluorescence from a monocysteine protein with IAANS attached only to Cys 84 appears to respond primarily to direct Ca^{2+} binding.

MATERIALS AND METHODS

Protein Engineering. A bacterial expression vector for cTnC was described previously (Putkey et al., 1989). All mutagenesis was performed by the method of Kunkel (1985) using the BioRad Mutagene kit. Recombinant proteins were overproduced in the *Escherichia coli* strain JM109 containing the Kan^r plasmid pGp1−2 encoding the temperature sensitive repressor cI857 under the control of the lac promoter. All proteins were isolated as described previously (Putkey et al., 1989). The predicted sulfhydryl content of the mutant proteins was confirmed by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Descriptive nomenclature for the mutated proteins is given in Figure 1.

Fluorescence Labeling. Protein solutions were first made 2 mM in EDTA and 7.5 mM in DDT and then exhaustively dialyzed against 50 mM Tris, pH 7.5, 150 mM KCl, 0.2 mM DTT, 0.2 mM EDTA. The dialyzed proteins were adjusted to a concentration of 1−2 mg/mL and used directly, or made 6 M in urea prior to labeling. Labeling was initiated by the addition of IAANS at a concentration which was sufficient to saturate the DTT and be in 4-fold molar excess over Cys residues. The labeling reaction was allowed to proceed for 2−5 h in the dark at room temperature and then terminated by the addition of Cys to a final concentration of 10 mM. Unreacted IAANS and Cys were removed by either dialysis or desalting into 50 mM Tris, pH 7.5, 150 mM KCl, with or without 6 M urea. Proteins that were labeled and processed in the presence of urea were further dialyzed against 50 mM Tris, pH 7.5, 150 mM KCl. All protein concentrations were determined using the BCA assay (Pierce Chemicals) with purified cTnC3 as a standard. The amount of IAANS associated with the protein was determined by UV absorbance at 325 nm using an extinction coefficient of 24 900 M^{-1}. The extent of labeling was more variable in the

absence of urea. A comparison of Ca^{2+}-dependent fluorescence from proteins labeled in the absence or presence of urea showed essentially identical pCa_{50} values for fluorescence changes. Thus, the majority of proteins used in the current experiments were labeled in the presence of urea.

Steady state fluorescence measurements were made using a Perkin-Elmer LS-5 fluorescence spectrometer at excitation and emission wavelengths of 335 and 455 nm, respectively. Samples were prepared in a buffer of 50 mM MOPS, pH 7.0, 150 mM KCl, 5 mM DDT, 0.2 mM EGTA and 25 mg/mL protein. Magnesium was omitted to avoid dimerization of cTnC as reported previously (Jaquet & Heilmeyer, 1987). The concentration of total Ca^{2+} needed to achieve a desired free Ca^{2+} concentration was calculated using a computer program of Fabiato (1988) and an apparent association constant of EGTA for Ca^{2+} at pH 7.0 and 23 °C of 2.53×10^6. Using the above concentrations of MOPS and EGTA the pH of the solutions changed by a maximum of 0.04 upon the addition of excess Ca^{2+}. The relative fluorescence emission values were corrected for nonspecific effects of dilution and changes in ionic strength by titration of a second sample with equal volumes of isotonic KCl solutions.

Formation of Troponin Complexes. Formation of binary and ternary complexes was performed essentially as described by Potter (1982). Cardiac TnI and cTnT, in 6 M urea, 50 mM Tris, pH 8.0, were added to IAANS-labeled cTnC3 or monocysteine derivatives and then dialyzed against 1 M KCl, 10 mM MOPS, pH 7.0, 5 mM $MgCl_2$. The molar ratios of cTnI, cTnT, and cTnC were 2:2:1, respectively, to avoid dimer formation between cTnC and cTnI or cTnT. The protein solutions were sequentially dialyzed against the same buffer but with KCl concentrations of 0.7, 0.5, 0.3, and 0.1 M. After the final dialysis the protein solutions were centrifuged for 10 min at 0−4 °C in a microfuge. As judged by native and SDS−polyacrylamide gel electrophoresis, no free cTnC was present and excess cTnT and cTnI were removed by centrifugation. Titration of troponin complexes with Ca^{2+} was performed as for the monomeric proteins but in the presence of 5 mM $MgCl_2$ to stabilize the complexes. Preparation of binary complexes was identical to the preparation of the whole troponin complex except that only cTnI and cTnC derivatives were combined in a molar ratio of 2:1.

Reaction of Monocys Proteins with DTNB and CPM. Stock assay buffers were prepared which contained 50 mM MOPS, 200 mM KCl, 1 mM EGTA, with or without sufficient $CaCl_2$ to achieve pCa 6.8 or 4.4. The stock buffer for binary and ternary complexes contained 2 mM $MgCl_2$ to stabilize the complex. Magnesium was omitted from buffers used for the free proteins to avoid dimerization of cTnC (Jaquet & Heilmeyer, 1987). All stock buffers were adjusted to pH 7.0 after the addition of EGTA, $CaCl_2$, and $MgCl_2$. The stock buffers were diluted 2-fold with a solution of protein in 50 mM MOPS, pH 7.0 such that the final protein concentration was 5 μM for DTNB reactions and 1 μM for CPM. The reactions were initiated by the addition of DTNB and CPM to final concentrations of 250 and 25 μM, respectively. Similar to Fuchs et al. (1989), the rate of reaction was first order with respect to these reagents (data not shown).

Free sulfhydryl groups in cTnI and cTnT were blocked with *N*-ethylmaleimide prior to formation of binary and ternary complexes to be used for analysis with DTNB or CPM. cTnI and cTnT were dialyzed against 50 mM MOPS, pH 7.0, 6 M urea and reduced with 5 mM dithiothreitol. The protein solutions were then desalted into 50 mM MOPS, pH 7.0, 6 M urea, 1 mM *N*-ethylmaleimide and dialyzed against the same buffer without *N*-ethylmaleimide. Binary and ternary complexes were formed as described above. The absence of reactive sulfhydryl residues in blocked cTnI and cTnT was confirmed by performing control DTNB and CPM reactions with ternary and binary complexes formed with cTnC(A-Cys). cTnC and cTnI preparations were used for subsequent experiments with cTnC(C35) and cTnC(C84) only if their Cys residues were quantitatively blocked.

Measurements in Skinned Muscle Fibers. Skinned rat cardiac muscle fibers were prepared from the rat left ventricular muscle (Hoar et al., 1988), either by the method of Kerrick and Krasner (1975) or by dissection, and placed in a relaxing solution. The ends of the skinned fiber preparations were inserted into small stainless steel clamps of a tension transducer system (Guth & Wojciechowski, 1986). The preparations were then perfused with a relaxing solution containing 1% Triton X-100 for 20 min to solubilize any remaining sarcolemma and sarcoplasmic reticulum. This facilitates the diffusion of exogenous proteins into or out of the skinned fiber bundles. The cells were then ready to be immersed into various test solutions for mechanical and fluorescence measurements.

Standard solutions contained 85 mM K^+, 2 mM $MgATP^{2-}$, 1 mM Mg^{2+}, 7 mM EGTA, 10^{-8}−10^{-3} M Ca^{2+}, 15 mM creatine phosphate, 15 units of creatine phosphokinase/mL, and propionate as the major anion. The ionic strength was adjusted to 0.15, and pH was maintained at 7.00 ± 0.02 with imidazole propionate. Relaxing solutions had no added Ca^{2+}. Contracting solutions contained various concentrations of free Ca^{2+}. The concentrations of the various ionic species were determined by computer solution of ionic equilibrium equations using published binding constants (Donaldson & Kerrick, 1975).

Mechanical and optical measurements were made on small bundles of skinned (permeabilized) cardiac muscle cells attached to a force transducer and length controller which fit into a 1 mm^2 quartz capillary cuvette. The cuvette allows for the bathing of the skinned bundles of cells in test solutions and for optical measurements associated with the muscle fiber. Perfusion of the cuvette was controlled by an electrical valve and its control unit. The open time of the valve was adjusted between 0.1 and 1.0 s, and the repetition frequency of solution changes ranged between 2 and 5 times per minute. Both the repetition frequency and the duration of the valve open period were externally controlled by the computer. To this system was attached a gradient maker which allowed for the mixing of a maximal contracting solution (pCa = 3.0) with a relaxing solution (pCa = 9.0).

The muscle fiber in the cuvette was illuminated by UV light (320 nm) polarized at right angles to the fiber's axis. Polarized fluorescence was measured at right angles to the fiber axis using a microscope photometer system unit with an adjustable slit in the image plane. Polarization of the excitation and emission light was used in order to minimize light scatter. It was also determined that there was no fluorescence polarization signal. This was done by measuring simultaneously the fluorescence signals perpendicular and parallel to the fiber axis during contraction. The difference

of these two signals divided by their sum did not change during Ca^{2+} activation of the fiber.

Removal of the endogenous cardiac TnC from skinned cells was accomplished by exposing the bundle of fibers to a low ionic strength EDTA solution, using the method previously described for the purification of TnC (Cox et al., 1981) and subsequently used for the removal and replacement of TnC in myofibrils (Zot & Potter, 1982) and skinned skeletal (Kerrick et al., 1985) and cardiac (Hoar et al., 1988) muscle cells. After mounting the bundle of skinned cells in the apparatus, the fibers were first immersed in a wash solution (see above) for approximately 5 min, and then the TnC was extracted with the EDTA solution for approximately 45 min. Following the extraction procedure the fibers were immersed in the wash solution for 2 min before the preparation was immersed in a relaxing solution. The cell bundle was then test contracted to assess the extent of cTnC removal. Following this test contraction, the extracted bundle of cells was immersed into a relaxing solution containing 2 mg/mL recombinant IAANS-labeled cTnC for 20 min and another test contraction performed to assess the recovery state of the cell bundle. The fibers were then immersed in a relaxing solution, and the Ca^{2+} gradient was started.

RESULTS

Proteins. Figure 1 summarizes the nomenclature used here and provides a representation of the predicted secondary structure of cTnC. The mutant proteins were reported previously (Putkey et al., 1993) and assigned names to indicate the position of the reactive Cys residue. Thus, cTnC(C35) has a single Cys at residue 35 due to conversion of Cys 84 to either Ser or Ala. cTnC in which both Cys residues were converted to Ser is called cTnC(A-Cys). Proteins which have been reacted with IAANS are designated with the subscript IA, i.e., $cTnC(C84)_{IA}$. Unless otherwise indicated all data were collected using the Ser mutants.

Effect of Metal Binding on Fluorescence from IAANS-Labeled Proteins. The IAANS-labeled proteins were first used to determine if covalent modification of individual Cys residues with the fluorescent probe IAANS has an effect on protein structure which would adversely affect function. We reasoned that the relative Ca^{2+}-binding properties of the respective native and IAANS-labeled proteins would be a good indicator of such an event.

Previous studies using IAANS-labeled native (Johnson et al., 1980; Hannon et al., 1992) or recombinant cTnC (Putkey et al., 1989) showed that binding of Ca^{2+} or Mg^{2+} to sites III and (or) IV decreased fluorescence, while binding Ca^{2+} to site II increased fluorescence. Figure 2 shows the effect of Mg^{2+} on fluorescence from IAANS-labeled cTnC and monocys derivatives in which Cys 35 or Cys 84 was changed to Ser. All IAANS-labeled proteins showed a similar decrease in fluorescence in response to Mg^{2+} with an apparent K_d of about 3.2×10^{-4} M. Similar results were obtained for proteins in which Cys 35 or Cys 84 were changed to Ala (data not shown).

Figure 3 shows the effect of Ca^{2+} on fluorescence from IAANS-labeled proteins in the free form or after association with cTnI or cTnI and cTnT. The pCa_{50} values for these data are listed in Table 1. Titration of the free forms of all proteins with Ca^{2+} (Figure 3, panel A) resulted in an initial

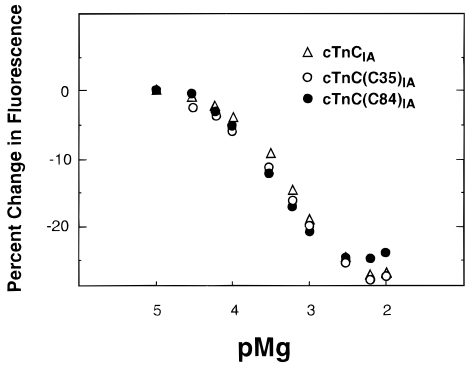

FIGURE 2: Effect of Mg^{2+} on fluorescence from IAANS-labeled proteins. The effect of the Mg^{2+} on steady state fluorescence from the free form of IAANS-labeled proteins is expressed as the percent change in fluorescence relative to the initial fluorescence value in arbitrary units.

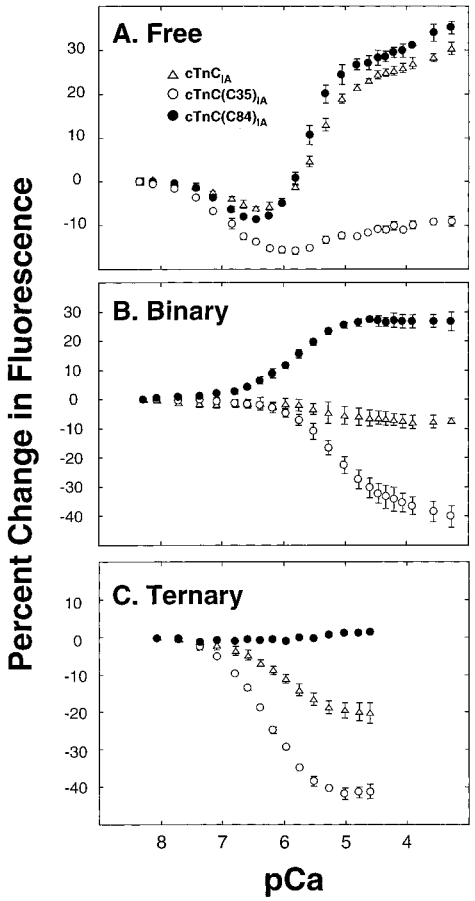

FIGURE 3: Effect of Ca^{2+} on fluorescence from IAANS-labeled proteins (A) in the free form, (B) in a binary cTnC·cTnI complex, and (C) in a ternary Tn complex. The effect of Ca^{2+} on steady state fluorescence is expressed as the percent change in fluorescence relative to the initial fluorescence value in arbitrary units. The error bars represent the standard deviation of three separate titrations. Buffer conditions are given in Materials and Methods.

decrease in fluorescence with a pCa_{50} of about 6.9 ($K_d \approx 1.4 \times 10^{-7}$ M). In all experiments, little or no Ca^{2+}-dependent change in fluorescence was seen between pCa 6 and pCa 4 for $cTnC(C35)_{IA}$ in which Cys 84 was converted to either Ser (see Figure 3, panel A) or Ala (data not shown). Increasing the concentration of free Ca^{2+} from pCa 6.5 to 5 resulted in an increase in fluorescence from both $cTnC_{IA}$ and $cTnC(C84)_{IA}$ with a pCa_{50} of about 5.5 ($K_d \approx 3.2 \times 10^{-6}$ M; see Table 1). The pCa_{50} values for the high- and low-

Table 1: Comparison of pCa_{50} Values for IAANS-Labeled Proteins[a]

protein	free protein		binary complex	ternary complex	skinned fibers	
	low	high			fluorescence	force
$cTnC_{IA}$	5.46 ± 0.10	6.83 ± 0.12	−	6.10 ± 0.05	5.67 ± 0.01	5.29 ± 0.04
$cTnC(C84)_{IA}$	5.56 ± 0.06	6.95 ± 0.10	5.90 ± 0.10	−	5.31 ± 0.04	5.29 ± 0.04
$cTnC(C35)_{IA}$[b]	−	6.77 ± 0.22	5.23 ± 0.09	6.30 ± 0.04	−	−
$cTnC(C35)_{IA}$[c]	−	6.96 ± 0.03	5.21 ± 0.22	6.26 ± 0.02	nd[d]	nd

[a] The pCa_{50} is defined as −log of the apparent dissociation constant for calcium. These values were derived from the data in Figures 3 and 4 using the equation $F = [F_{max1}/(1 + 10^{n_1[\log(KCa_1)-\log(Ca)]}] + [F_{max2}/(1+10^{n_2[\log(KCa_2)-\log(Ca)]}]$, where F is the fluorescence at a given free calcium concentration, F_{max} is the maximal fluorescence change, n is the Hill coefficient, Ca is the concentration of free calcium, and KCa is the apparent dissociation constant for calcium. All values represent the average ± SD of four independent titrations. Proteins and conditions which did not give a reproducible chance in fluorescence or could not be reliably fit to the Hill equation are indicated by −. Low and high refer to the low-affinity site II and the high-affinity sites III and IV, respectively. Binding to the high-affinity sites is observed only in the free forms of the proteins since the complexes are stabilized by saturation of sites III and IV with Mg^{2+}. [b] Monocysteine derivative with C84S mutation. [c] Monocysteine derivative with C84A mutation. [d] Not determined.

affinity sites in free TnC compare well with values reported previously (Johnson et al., 1980; Hannon et al., 1992; Holroyde et al., 1980; Putkey et al., 1993).

Figure 3, panel B, and Table 1 show that binary cTnC·cTnI complex formed with $cTnC(C84)_{IA}$ exhibits an increase in fluorescence with a pCa_{50} of 5.9. This is similar to the apparent K_d of 1×10^{-6} M (pCa_{50} 6.0) reported for the unlabeled binary complex (Holroyde et al., 1980). Surprisingly, binary complexes formed using $cTnC(C35)_{IA}$ showed a Ca^{2+}-dependent decrease in fluorescence with a very low apparent affinity of site II for Ca^{2+} with a pCa_{50} of about 5.2. This is not due to the nature of mutation at position 84 since it was also observed for IAANS-labeled cTnC(C35) in which Cys 84 was changed to either Ser or Ala (see Table 1). Binary complexes formed with $cTnC_{IA}$ showed a very small decrease in fluorescence upon Ca^{2+} binding to site II. This change was too small and variable to derive a reliable pCa_{50} value, but it appears that this change parallels that seen for complexes formed with $cTnC(35)_{IA}$ rather than $cTnC(C84)_{IA}$.

Figure 3, panel C, shows the effect of Ca^{2+} on fluorescence emissions from ternary troponin complexes. There was no reproducible Ca^{2+}-dependent change in fluorescence from ternary complexes formed with $cTnC(C84)_{IA}$. Ternary complexes formed with $cTnC_{IA}$ or $cTnC(C35)_{IA}$ showed a Ca^{2+}-dependent decrease in fluorescence with pCa_{50} values of 6.1 and 6.3, respectively (see Table 1). These values are comparable to the apparent K_ds of 4.0×10^{-7} M (pCa_{50} 6.4) for native troponin (Holroyde et al., 1980) and 3.3×10^{-7} (pCa_{50} 6.5) for ternary troponin complex formed with IAANS-labeled cTnC (Johnson et al., 1980). Thus, the unusually low affinity of site II for Ca^{2+} in the binary complex of $cTnC(C35)_{IA}$·cTnI is apparently corrected upon formation of the ternary complex.

Reactivity of Cys Residues with DTNB and CPM. The results in Figure 3 show that Ca^{2+} binding to site II in the binary complex of cTnC(C35)·cTnI is influenced either by mutation of Cys 84 to Ser or Ala or by covalent modification of Cys 35 with IAANS. To distinguish between these mechanisms, and to gain more insight into the relative effects of Ca^{2+} and other troponin subunits on the microenvironment of the Cys residues, we chose to determine the relative reactivity of the sulfhydryl groups with DTNB and CPM. The rate of covalent modification of free sulfhydryl groups with these reagents is dependent on the relative accessibility and the chemical environment of the Cys residue. These reagents have been used previously to study the reactivity of Cys residues in native free cTnC (Fuchs et al., 1989).

Table 2: Pseudo-First-Order Rate Constants for the Reaction of cTnC(C35) and cTnC(C84) with DTNB or CPM[a]

protein	reagent	pCa	pseudo-first-order rates ($s^{-1} \times 10^2$)		
			free	C·I	C·I·T
cTnC(C35)	DTNB	6.8	6.2 ± 0.3	28 ± 0.6	14 ± 0.6
		4.4	7.7 ± 0.4	2.1 ± 0.2	3.9 ± 0.4
	CPM	6.8	2	25	16
		4.4	2	4	5
cTnC(C84)	DTNB	6.8	4.3 ± 0.2	43 ± 2	71 ± 8
		4.4	6.2 ± 0.4	64 ± 2	82 ± 1
	CPM	6.8	8	73	67
		4.4	45	80	48

[a] Experimental data was fit the equation $A = A_{max}(1 - e^{-kt})$. Values for DTNB represent the average ±SD of three separate experiments. Experimental conditions are described in Materials and Methods.

Preliminary experiments showed that the time course of reaction of DTNB or CPM with all forms of the monocys proteins could be fit to a monoexponential equation, and that Ca^{2+}-dependent changes in the rate of reaction were due to saturation of site II (data not shown). Table 2 shows the pseudo-first-order rate constants obtained at pCa 6.8 when site II is predominately in the apo form, and at pCa 4.4 when site II is in the Ca^{2+}-bound form. The free forms of both cTnC(C35) and cTnC(C84) showed similar rates of reaction with DTNB which were slightly increased by Ca^{2+} binding to site II. In contrast, Ca^{2+} binding to site II increased the rate of reaction of CPM with cTnC(C84) about 6-fold, but had no effect on cTnC(C35).

Association of cTnC(C84) with cTnI or cTnI/cTnT caused a significant increase in the rate of reaction of both DTNB and CPM with Cys 84, but reaction rates were only slightly affected by Ca^{2+} binding to site II. Association of cTnC(C35) with cTnI or cTnT/cTnI in the absence of Ca^{2+} caused a significant increase in the rate of reaction of both DTNB and CPM with Cys 35. In contrast to the relative reactivity of Cys 84, Ca^{2+} binding to site II caused a significant decrease in the rate of reaction of both DTNB and CPM with Cys 35 when cTnC(C35) is associated with either cTnI or cTnI/cTnT. This suggests a Ca^{2+}-dependent change in the microenvironment of Cys 35 but not Cys 84, in the binary or ternary complex.

Figure 4, panel A, shows the results of an experiment in which we determined the time course of reaction of cTnC(C35)·cTnI with DTNB at various free Ca^{2+} concentrations. A pCa_{50} of 6.3 and a Hill coefficient of 0.75 were derived by plotting the rate of reaction versus the concentration of free Ca^{2+} (Figure 4, panel B). This is much greater than

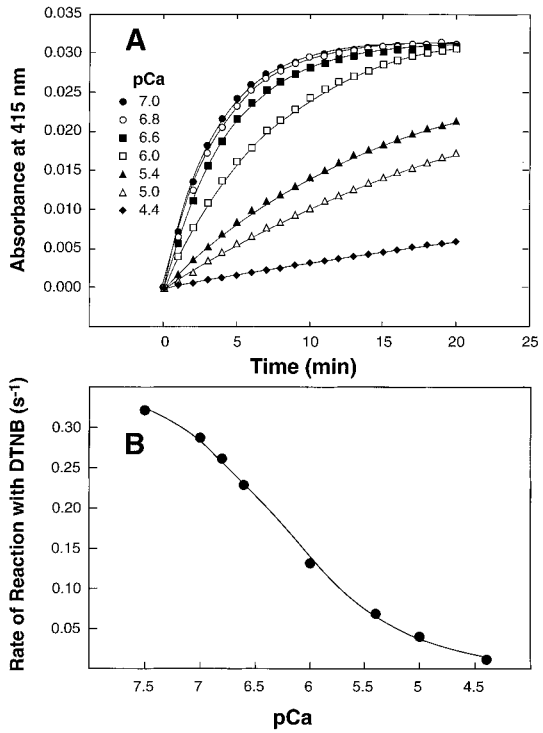

FIGURE 4: Rate of reaction of DTNB with binary cTnC(C35)$_{IA}$·cTnI complexes: (A) time course of reactions performed at the indicated free Ca^{2+} concentrations; (B) rate of reaction versus free Ca^{2+} concentration. Pseudo-first-order rate constants were determined as described in Table 2. A pCa_{50} of 6.3 was obtained by fitting the data in panel B to the equation shown in Table 1.

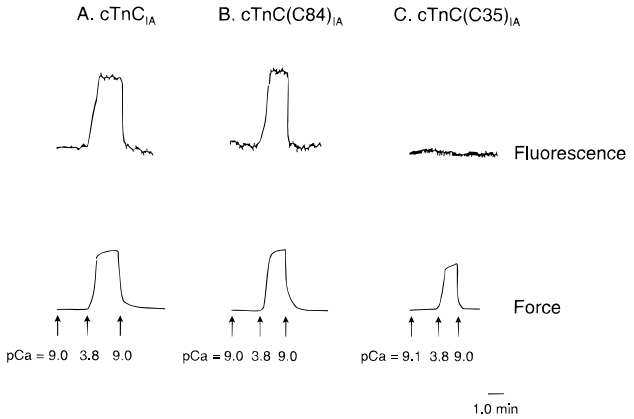

FIGURE 5: Fluorescence changes associated with cycling cross bridges and rigor in skinned cardiac fibers reconstituted with IAANS-labeled proteins. Skinned cardiac muscle fibers were depleted of endogenous cTnC and reconstituted with (A) cTnC$_{IA}$, (B) cTnC(C35)$_{IA}$, or (C) cTnC(C84)$_{IA}$. The fibers were then subjected to a series of relaxing (pCa 9.0) and contracting (pCa 3.8) solutions as indicated by the arrows. Force and fluorescence were monitored simultaneously as described in Materials and Methods.

the pCa_{50} of 5.2 seen for the binary complex of cTnC(C35)$_{IA}$·cTnI (Table 1). Thus it would appear that covalent modification of cTnC(C35) with IAANS, rather than the C84S mutation, affects the Ca^{2+}-binding properties of the protein, but only when it is associated with cTnI.

Ca^{2+} Dependence of IAANS-Labeled cTnC in Skinned Fibers. Figure 5 shows the effect of maximal contracting (pCa 3.8) and relaxing (pCa 9.0) solutions on fluorescence and force in skinned cardiac fibers containing either cTnC$_{IA}$, cTnC(C35)$_{IA}$, or cTnC(C84)$_{IA}$. Only cTnC$_{IA}$ and cTnC(C84)$_{IA}$ showed a detectable change in fluorescence during

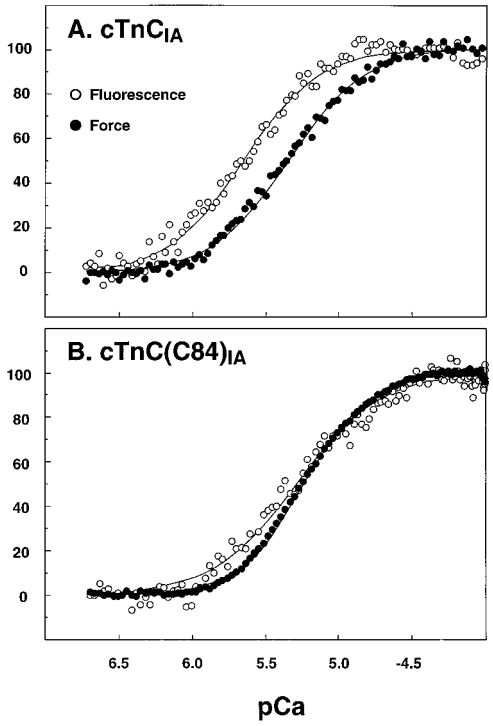

FIGURE 6: Correlation of force and fluorescence from skinned cardiac fibers. Force and fluorescence in cTnC-extracted skinned cardiac muscle fibers reconstituted with either cTnC$_{IA}$ (A) or cTnC(C84)$_{IA}$ (B) were simultaneously monitored as a function of increasing free Ca^{2+} concentrations. Changes are expressed as a percent of maximal response.

maximal contraction. This increase in fluorescence is in contrast to the Ca^{2+}-dependent decrease in fluorescence seen for cTnC$_{IA}$ associated with a ternary troponin complex (see Figure 3, panel C). In other experiments, all IAANS-labeled proteins showed an increase in fluorescence when rigor was induced in the fibers in the absence of Ca^{2+} (data not shown). The lack of fluorescence change from cTnC(C35)$_{IA}$ during Ca^{2+}-activated cycling cross bridges may be due to (1) no change in the environment surrounding Cys 35 upon Ca^{2+} binding to site II of cTnC or (2) cycling cross-bridges inducing a conformational change in the region of Cys 35 which cannot be detected by the IAANS probe.

Figure 6 shows representative pCa vs force and fluorescence curves for TnC-extracted cardiac muscle fibers that were reconstituted with either cTnC$_{IA}$ (panel A) or cTnC(C84)$_{IA}$ (panel B). Both fibers showed similar pCa_{50} values for force generation (see Table 1). However, fluorescence preceded force in fibers that were reconstituted with cTnC$_{IA}$. Extracted cardiac fibers reconstituted with cTnC(C84)$_{IA}$ had essentially identical pCa_{50} values for force and fluorescence.

Several conditions were tested to determine the effect of force producing cross bridges on fluorescence. Figure 7 shows the effect of vanadate on the Ca^{2+} dependence of fluorescence from fibers reconstituted with cTnC$_{IA}$ or cTnC(C84)$_{IA}$. In both fibers vanadate inhibited force by about 80% without decreasing the maximal fluorescence. Vanadate had little effect on the pCa_{50} of fluorescence from fibers reconstituted with cTnC(C84)$_{IA}$. However, it caused a rightward shift (higher Ca^{2+} concentration) of about 0.4 in the pCa_{50} of fluorescence from fibers that were reconstituted with cTnC$_{IA}$.

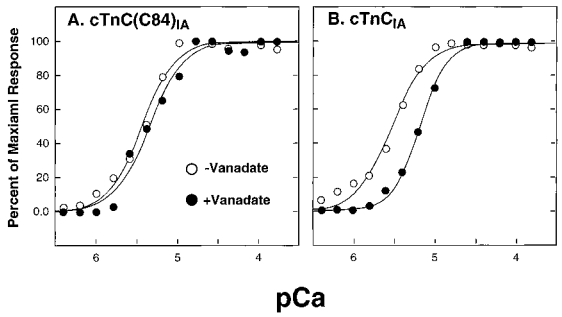

FIGURE 7: Effect of vanadate on Ca^{2+}-dependent fluorescence. Skinned cardiac muscle fibers were depleted of endogenous cTnC and reconstituted with $cTnC_{IA}$ (A) or $cTnC(C84)_{IA}$ (B). Fluorescence was monitored as a function of increasing free Ca^{2+} concentrations.

DISCUSSION

The recent solution NMR structure for cTnC[2] shows that Cys 35 and Cys 84 are on opposite sides of the N-terminal globular domain. We reasoned that these different environments may allow attached probes to sense different events in the thin filament, but they may also provide different potential for functional perturbation induced by covalent modification. For example, Cys 35 in the inactive loop I resides in a short β strand between loops I and II. Coupling a rather large probe at this location could have direct adverse effects on Ca^{2+} binding to cTnC. Thus it was important that we first characterize the biochemical properties of monocysteine derivatives of cTnC and their fluorescent labeled derivatives so that they can be used as reliable fluorescent markers.

The relative reactivity of the monocys mutants with DTNB and CPM is consistent with previous studies using native cTnC and suggests that the amino acid changes themselves do not alter structure. The similarity of the rates of reaction of DTNB with cTnC(C35) and cTnC(C84) in the presence and absence of Ca^{2+} explains why Fuchs et al. (1989) observed a single rate constant for native cTnC. Binding Ca^{2+} to site II induced a slight increase in the rate of reaction of CPM with cTnC(C35) but a marked increase in the rate of reaction with cTnC(C84). This is also consistent with Fuchs et al. (1989), who concluded that a biphasic reaction of CPM with cTnC was due to a Ca^{2+}-dependent increase in the rate of reaction with Cys 84, but not Cys 35.

Association of cTnC(C35) or cTnC(C84) with cTnI and cTnT caused significant changes in the reactivity of the two Cys residues with CPM and DTNB. The most interesting observation was that the rate of reaction of Cys 35 is significantly decreased when Ca^{2+} binds to site II of cTnC(C35) bound to either cTnI or cTnI/cTnT. This was not observed for the free protein and suggests that association of cTnC with the other troponin subunits has specific effects on localized Ca^{2+}-dependent changes in the inactive loop I. This concept is supported by data in Figures 3 and 4 and Table 2 which show that the Ca^{2+}-binding affinity of site II in $cTnC(C35)_{IA}$, but not cTnC(C35), is significantly decreased upon association with cTnI. This effect of covalent modification was not observed for $cTnC(C84)_{IA}$. We cannot discern if the aberrant Ca^{2+}-binding property persists in $cTnC_{IA}$ since no reliable, significant change in fluorescence was observed for $cTnC_{IA}$·cTnI. Previous studies using IAANS-labeled cTnC did not perform Ca^{2+} titrations of the binary complex (Johnson et al., 1980; Liao et al., 1994; Dong et al., 1996).

The importance of the inactive loop I in cTnC, which contains Cys 35, should be emphasized. Sheng et al. (1990) proposed that the inactive site I of cTnC somehow allows site II to be an effective regulator of muscle contraction. Amino acids Cys 35 and Cys 84 can spontaneously form an intramolecular disulfide bond (Putkey et al., 1992), and that this renders the protein Ca^{2+} independent when associated with skeletal muscle myofibrils (Putkey et al., 1993) or skinned fibers (Hannon et al., 1993). The precise structural basis for the effect of IAANS attached to Cys 35 is not clear, but it may involve constrained movement of the N-terminal β strands when IAANS-labeled cTnC(C35) is associated with cTnI. Such movement in native cTnC is suggested by Ca^{2+}-dependent strengthening of a hydrogen between these β strands (Krudy et al., 1992). Low-angle X-ray scattering predicts that a region of sTnI is positioned near the N-terminal β strands in sTnI (Olah et al., 1994). The negatively charged sulfonic acid group of IAANS bound to Cys 35 may form a salt bridge with the basic cTnI and hinder proper juxtaposition of the two β strands. It is not clear why an effect of IAANS bound to Cys 35 is observed for the $cTnC(C35)_{IA}$·cTnI and not $cTnC(C35)_{IA}$·cTnI·cTnT.

The most interesting feature of fluorescence from IAANS-labeled proteins incorporated into skinned fibers is that the pCa_{50} values of fluorescence and force are coincident for fibers containing $cTnC(C84)_{IA}$, yet the pCa_{50} for fluorescence occurs at a lower Ca^{2+} concentration than the pCa_{50} for force for fibers containing $cTnC_{IA}$. We cannot at present provide a precise mechanism to account for why fluorescence for IAANS-labeled cTnC precedes force, but there are at least four possibilities. The first general mechanism involves undesirable structural differences between IAANS-labeled cTnC and cTnC(C84). We cannot rule out this possibility, especially since coupling IAANS to Cys 35 alters Ca^{2+} binding to site II of $cTnC(C35)_{IA}$ when bound to cTnI. But this is not observed when $cTnC(C35)_{IA}$ is associated with both cTnI and cTnT as it would be in the thin filament. Moreover, the pCa_{50} of force generation is the same for fibers reconstituted with $cTnC(C84)_{IA}$ or $cTnC_{IA}$. Distinct force pCa_{50} values may be anticipated if the basal Ca^{2+} affinity of site II for the two proteins were different, similar to what is seen for skeletal or cardiac preparations reconstituted with cTnC or sTnC (Moss et al., 1986; Morimoto & Ohtsuki, 1988).

The second general mechanism is that the two IAANS-labeled proteins sense different Ca^{2+}-dependent events in the skinned fibers such as non-force-generating cross bridges, force-generating cross-bridges, thin filament cooperativity, or direct Ca^{2+} binding. An effect of cross bridges on fluorescence is suggested by several observations. The first studies using sTnC labeled at Met 25 with the fluorescent probe DANZ (Zot et al., 1986; Guth & Potter, 1987) showed that fluorescence was activated at a lower Ca^{2+} concentration than force. These studies also showed that fluorescence could be affected by rigor and cycling cross bridges. Cycling cross bridges had the greatest effect on fluorescence, and this was due to a state in the cross-bridge cycle that was not

[2] Sia, S. K., Li, M. X., Spyracopalous, L., Gangne, S. M., Liu, W., Putkey, J. A. and Sykes, B. D. (submitted).

analogous to the rigor cross bridge. Kerrick et al. (1991), using skeletal fibers reconstituted with DANZ-labeled sTnC, also showed that fluorescence preceded force but that the Ca^{2+} dependence of ATPase and fluorescence were more tightly coupled. This suggests coupling between cross-bridge formation and fluorescence. Thus, it is possible that fluorescence from IAANS-labeled wild-type cTnC is sensitive to cross-bridge attachment or cycling cross bridges. This mechanism is similar to that proposed by Hannon et al. (1992), who showed that fluorescence from IAANS-labeled cTnC incorporated into skinned skeletal and cardiac fibers more closely parallels stiffness than force and both fluorescence changes and stiffness are activated at a lower Ca^{2+} concentration than force. Fluorescence from IAANS-labeled cTnC(C84) may be predominately affected by Ca^{2+}-dependent changes in conformation that are tightly coupled to force generation.

A third explanation for the apparent higher Ca^{2+} sensitivity of fluorescence from $cTnC_{IA}$ is that this doubly-labeled protein responds to both direct Ca^{2+} binding and cooperativity along the thin filament, mediated through Ca^{2+}-activated myosin cross bridges or adjacent troponin/tropomyosin units. In other words, binding Ca^{2+} to one $cTnC_{IA}$ could cause several neighboring labeled apo cTnCs to increase their fluorescence to varying degrees depending upon how far they were from the cTnC with the Ca^{2+} bound. Such cooperativity would lead to an apparent half-maximal increase of fluorescence at a lower Ca^{2+} concentration than half-maximal Ca^{2+} binding to the thin filament or activation of force. Vanadate was recently shown by X-ray diffraction to cause detachment of myosin heads from the thin filament regardless of the Ca^{2+}-bound state of the thin filament (Takemori et al., 1995). The fact that vanadate cause a rightward shift in the pCa vs fluorescence curve suggests that myosin cross bridges play a role in the putative cooperative effect and that fluorescence from $cTnC_{IA}$ can be increased by Ca^{2+} in the absence of strong cross-bridge formation, presumably due to direct Ca^{2+} binding.

The fourth possibility arises from recent studies of Dong et al. (1996), who used cTnC labeled with IAANS at Cys 84 to study the kinetics of Ca^{2+} binding to site II of free cTnC. They reported two distinct fluorescence transients. One was fast and with a rate that was dependent on the concentration of Ca^{2+}, while the other was slow and independent of Ca^{2+} concentration; the latter they attributed to movement of helices in the N-terminal regulatory domain. It is difficult to confidently relate these kinetics studies of the free protein with the current steady state measures reported here using IAANS-labeled cTnC in skinned fibers. Nevertheless, the concept that Ca^{2+} binding and conformational changes in cTnC are temporally separable events in the free protein presents an intriguing hypothesis which could account for the different pCa_{50}s of fluorescence for IAANS-labeled cTnC and cTnC(C84) seen in Figure 6. This hypothesis would invoke the possibility that Ca^{2+} binds to cTnC but that constraints imposed on cTnC within the context of the thin filament may prevent a triggering conformational change in the regulatory domain to occur, even under steady state conditions, until a second condition is attained. This second condition may involve cooperative events mediated by tropomyosin or by myosin cross bridges. Thus, Ca^{2+} binding to site II may "prime" cTnC without initiating a conformational change that is necessary for forcegeneration. If IAANS-labeled cTnC were sensitive primarily to this priming event, then Ca^{2+}-dependent fluorescence changes would precede force. If IAANS-labeled cTnC(C84) were sensitive to the major triggering conformational change, then its fluorescence would be coincident with force.

In summary, two monocysteine derivatives of cTnC which can be labeled specifically with IAANS at Cys 35 or Cys 84 have been characterized with respect to their reaction rate with sulfhydryl reagents and fluorescent properties of IAANS-labeled cTnC *in vitro* and *in situ.* The data show that Ca^{2+}-dependent fluorescence from the monolabeled proteins is dependent upon the position of the label, other troponin subunits, and protein−protein interactions *in situ*. Most importantly these data show that, in contrast to wild type IAANS-labeled cTnC with two bound probes, the monocysteine cTnC with IAANS bound only to Cys 84 may be a very useful fluorescence probe for measuring Ca^{2+} binding to free cTnC and its complexes with cTnI and cTnT *in vitro. In situ*, IAANS-labeled cTnC(C84) is the most suitable probe for measuring coupled Ca^{2+}-dependent conformational changes in cTnC that result in activation of skinned fibers. Therefore these monocysteine derivatives of cTnC show great promise of providing investigators with new tools for investigating the interaction of Ca^{2+}, troponin subunits, and other proteins with cTnC *in vitro* and *in situ*.

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