Disparate Fluorescence Properties of 2-[4′-(Iodoacetamido)anilino]-Naphthalene-6-Sulfonic Acid Attached to Cys-84 and Cys-35 of Troponin C in Cardiac Muscle Troponin

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ABSTRACT Two monocysteine mutants of cardiac muscle troponin C, cTnC(C35S) and cTnC(C84S), were genetically generated and labeled with the fluorescent probe 2-[4′-(iodoacetamido)anilino]naphthalene-6-sulfonic acid (IAANS) at Cys-84 and Cys-35, respectively. Cys-84 is located on helix D in the regulatory N-domain, and Cys-35 is at the $-y$ position of the inactive 12-residue loop of site I. These labeled mutants were studied by a variety of steady-state and time-resolved fluorescence methods. In the absence of divalent cation, the fluorescence of the attached IAANS indicated an exposed environment at Cys-35 and a relatively less-exposed environment at Cys-84. The binding of Ca^{2+} to the single regulatory site elicited a large enhancement of the emission of IAANS attached to Cys-84, but only marginal fluorescence changes of the probe at Cys-35. Upon reconstitution of the labeled cTnC mutants with troponin I and troponin T to form the three-subunit troponin, the fluorescence of IAANS-Cys-84 in apo-troponin was spectrally similar to that observed with the Ca^{2+}-loaded uncomplexed cTnC mutant. Only very moderate changes in the fluorescence of IAANS-Cys-84 were observed when the regulatory site in reconstituted troponin was saturated. The exposed Cys-35 environment of the uncomplexed cTnC mutant became considerably less exposed and less polar when the mutant was incorporated into apo-troponin. In contrast to the Cys-84 site, saturation of the regulatory site II by Ca^{2+} in reconstituted troponin resulted in a reversal of the environment of the Cys-35 site toward a more exposed and more polar environment. These results indicated involvement of the inactive loop I in the Ca^{2+} trigger mechanism in cardiac muscle. The fluorescence of IAANS at both Cys-84 and Cys-35 was sensitive to phosphorylation of cTnI in reconstituted troponin, and the sensitivity was observed with both apo-troponin and Ca^{2+}-loaded troponin.

INTRODUCTION

Troponin C (TnC) is the Ca^{2+}-binding protein of the thin filament of muscle and has a regulatory role in vertebrate skeletal and cardiac muscle. Ca^{2+}-induced conformational changes in TnC are believed to be transmitted via the linkage between TnC and TnI (troponin I) within the three-subunit troponin complex to activate actomyosin ATPase and switch on muscle contraction (Cheung et al., 1987). The crystal structures of TnC from skeletal muscle of turkey (Herzberg and James, 1988) and chicken (Stayshur et al., 1988) reveal a dumbbell-shaped molecule with two globular domains connected by a long central α-helix. Each domain contains two metal ion binding sites, designated as sites I and II in the N-domain, and sites III and IV in the C-domain. Sites III and IV have a relatively high affinity for Ca^{2+} ($K_a \sim 10^7\ M^{-1}$) and also bind Mg^{2+} competitively ($K_a \sim 10^3\ M^{-1}$), whereas sites I and II have a lower Ca^{2+} affinity and are specific for Ca^{2+} ($K_a \sim 10^5\ M^{-1}$). Current evidence indicates that the C-domain in which the two binding sites are occupied by Ca^{2+}/Mg^{2+} throughout the contraction/relaxation cycle plays a structural role, and the N-domain plays a regulatory role.

Cardiac TnC (cTnC) differs from skeletal TnC in that site I in the cardiac isoform is inactive in chelating Ca^{2+} due to several amino acid substitutions in critical positions in the 12-residue Ca^{2+}-binding loop (van Eerd and Takahashi, 1976). Although the three-dimensional structure of cTnC has not been determined, it is reasonable to assume, on the basis of sequence homology and similarities in physiologic functions, that its structure is similar to that of skeletal TnC. Reversible Ca^{2+} binding to site II is believed to induce conformational changes in the N-domain, and these changes appear to modulate the TnC-TnI linkage in the regulatory mechanisms. The precise nature of these conformational changes is still obscure. A useful biophysical approach to delineate Ca^{2+}-induced conformational changes in TnC is to use extrinsic fluorescent probes attached to different regions of the protein. Cardiac TnC contains two cysteine residues; Cys-35, located at the nonfunctional Ca^{2+}-binding loop I, and Cys-84, located at the C-terminal end of helix D, where the TnC-TnI interaction occurs. In previous studies, both Cys-35 and Cys-84 in native cTnC were modified with the fluorescent probe: 2-[4′-(iodoacetamido)anilino]naphthalene-6-sulfonic acid (IAANS), and the fluorescence of the probe was used to study the binding of Ca^{2+} to site II in isolated cTnC and in troponin reconstituted with the IAANS-labeled cardiac TnC (Johnson et al., 1980; Robertson et al., 1982; El-Saleh and Solaro, 1987), and the equi-

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librium interaction between cTnC and cTnI (cardiac troponin I) (Liao et al., 1994). In these previous studies, a 1.8- to 2-fold increase in the fluorescence of $cTnC_{IAANS}$ was observed when Ca^{2+} bound to site II in isolated cTnC. It has been assumed that the observed large increase in fluorescence is due predominantly to the probe attached to Cys-84, because Cys-84 is strategically located in the amino-terminal domain, and a probe linked to this position might sense Ca^{2+}-activated conformational changes involving specific helices. Studies of monocysteine cTnC mutants labeled at either Cys-84 or Cys-35 have confirmed this notion (Zhang et al., 1992; Dong et al., 1996). Cardiac troponin reconstituted with native cTnC doubly labeled with IAANS at both Cys-84 and Cys-35 showed a 15–20% decrease in fluorescence upon binding Ca^{2+} at site II (Johnson et al., 1980; Robertson et al., 1982; Liao et al., 1994). It is not entirely clear whether the observed fluorescence decrease in the troponin complex is due to the probe attached to both Cys-84 and Cys-35 or due predominantly to the probe attached to one or the other cysteine. The present work was initiated to characterize the fluorescence of IAANS individually linked to Cys-35 and Cys-84 and to study structural alterations in the regions of the two cysteine residues that are accompanied by Ca^{2+} activation in cardiac troponin. This was accomplished by using monocysteine mutants of cTnC, each labeled with IAANS at its single sulfhydryl group, and reconstituted with the other two cardiac troponin subunits. IAANS is an environmentally sensitive probe. The change in the fluorescence parameters of the probe attached to cTnC can be used to reveal changes in local conformation, particularly environmental changes in the vicinity of the attached probe. This information is used to gain insight into possible involvement of different segments of cTnC in calcium activation.

MATERIALS AND METHODS

Preparation of troponin subunits

Cardiac muscle troponin was extracted from an ether powder that was prepared from the left ventricles of fresh bovine hearts (Potter, 1982). Troponin subunits were initially separated on a CM-Sephadex C-50-120 column in the presence of 6 M urea, 50 mM Tris at pH 8.0, and 1 mM DTT. Fractions of crude cTnC, cTnI, and cTnT (cardiac troponin T), were pooled and subsequently purified, separately, on a DEAE-Sephadex A-50 column in the presence of 6 M urea. cTnC was eluted with a gradient from 0 to 0.5 M KCl at pH 7.0, while cTnI and cTnT were eluted at pH 8.0 with a gradient from 0 to 0.5 KCl. The purity of each protein was monitored by $NaDodSO_4$ polyacrylamide gel electrophoresis. The purified proteins were lyophilized in the presence of 0.3 M KCl, 0.5 mM EGTA, 0.5 mM DTT, 20 mM imidazole at pH 7.2, and stored at −20°C.

Preparation and characterization of cardiac TnC mutants

Mutants of cTnC from rat cardiac muscle were obtained using the expression vector pET-24a(+) and overexpressed in *Escherichia coli* BL21(DE3) as previously described (Dong et al., 1996). The ability of the mutants to support Ca^{2+} regulation of tension development was monitored using chemically skinned reconstituted rabbit skeletal muscle fibers (Martyn and Gordon, 1988). Mutant cTnC(C84S) and cTnC(C35S) were found to have good functional properties (Dong et al., 1996).

Preparation of labeled cTnC mutants

Mutants cTnC(C35S) and cTnC(C84S) were first dialyzed against 0.5 mM DTT in a solution containing 0.2 M KCl, 30 mM MOPS at pH 7.0, followed by a second dialysis in which DTT was omitted. The sulfhydryl-reduced protein was reacted with a two to three-fold molar excess of IAANS in the presence of 6 M urea at 4°C for 10 h. The reaction was terminated with a three to fivefold excess of DTT, and the solution was exhaustively dialyzed against 6 M urea, 30 mM MOPS at pH 7.0, 0.2 M KCl, and 2 mM EGTA at 4°C, to remove unreacted fluorophore and DTT. A further dialysis against the same buffer without urea was repeated three times. The concentration of the labeled protein was determined with either a turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen and Dobryszcka, 1959) or the Bradford method (Bradford, 1976), and the amount of label covalently attached to the protein was determined by absorbance, using a molar extinction coefficient of 24,900 $M^{-1}cm^{-1}$ (Johnson et al., 1980). The labeling ratio was >0.9 mol fluorophore/mol protein for the two monocysteine mutants, and 1.8–2.0 for native cTnC.

Reconstitution of troponin

Binary complex formed between IAANS-labeled mutant cTnC and native cTnI and ternary complex of labeled cTnC with native cTnI and cTnT were prepared by incubating the labeled cTnC with a large excess of cTnI or cTnI plus cTnT in a buffer containing 30 mM MOPS, pH 7.2, 1 mM DTT, 50 mM Ca^{2+}, and 6 M urea. After a 30-min incubation at room temperature, the solutions were first dialyzed at 4°C against a buffer containing 30 mM MOPS at pH 7.2, 1 mM DTT, 50 mM Ca^{2+}, 3 M urea, and 1 M KCl. The urea and KCl concentrations were then reduced stepwise by changing to the following dialysis buffers containing no urea and a decreasing level of KCl: a) 1.0 M KCl, b) 0.7 M KCl, c) 0.5 M KCl, d) 0.3 M KCl, and e) 0.1 M KCl. Uncomplexed cTnI and cTnT were precipitated during dialysis and were removed by centrifugation. The solutions containing the complexes were dialyzed against a final buffer containing 30 mM MOPS at pH 7.2, 1 mM DTT, 1 mM EGTA, and 0.3 M KCl (basic buffer).

Phosphorylation of cTnI

cTnI was phosphorylated at pH 7.0 in 50 mM KH_2PO_4, 0.5 mM EGTA, 0.5 mM DTT, and 125 units catalytic subunit of protein kinase A/mg cTnI. The reaction was started by adding ATP to a final concentration of 0.5 mM, followed by incubation at 30°C for 20 min. The solution was then dialyzed exhaustively at 4°C against the basic buffer.

Steady-state fluorescence measurements

Steady-state fluorescence measurements were carried out at 20 ± 0.1°C on an SLM 8000C spectrofluorometer. The band pass of both the excitation and emission monochromators was set at 3 nm, and the measurements were made in the ratio mode. Emission spectra were corrected for variations of the detector system with wavelengths. Quantum yields of IAANS were determined by the comparative method (Parker and Rees, 1960), using quinine sulfate in 0.1 N H_2SO_4 as the standard (0.52 at λ_{ex} = 365 nm and 0.47 at λ_{ex} = 325 nm) (Melhuish, 1964). Absorption spectra were recorded on a Beckman DU-40 spectrophotometer at room temperature.

Quenching of IAANS fluorescence intensity was measured by adding aliquots of an 8-M acrylamide to the sample. When necessary, a correction was made for inner filter effects. The emission was measured at 459 nm for nonphosphorylated sample and at 467 nm for phosphorylated samples with 325 nm excitation. The quenching data were analyzed by the Stern-Volmer equation. The dynamic bimolecular quenching constant (k_q) was calculated

from $k_q = K_{SV}/\langle\tau\rangle$, where K_{SV} is the Stern-Volmer dynamic quenching constant and $\langle\tau\rangle$ is the mean fluorescence lifetime.

A standard calcium solution (Orion) was used in titration experiments. EGTA was used to control the level of free calcium, and free calcium concentrations were calculated as described previously (Dong et al., 1996), using known stability constants of the chelator for proton and cations.

Time-resolved fluorescence measurements

Fluorescence intensity decay of IAANS covalently linked to mutant cTnC was measured in the basic buffer at 20°C on a PRA single photon-counting system (Model 3000) with a DCM [4-(dicyanomethylene)-2-methyl-6-(*p*-dimethylaminostyryl)-4H-pyran] dye laser synchronously pumped by a mode-locked argon ion laser (Model 171, Spectra-Physics). The mode-locker operated at 41 MHz, and the cavity-dumped laser was set at 4 MHz and provided a train of light pulses with a 15-ps full width at half-maximum (FWHM). The output from the dye laser was frequency-doubled to 325 nm by an angle-tuned KDP crystal (Model 390, Spectra-Physics) to generate uv/ps laser pulses with tunable frequency from 315 to 335 nm. The laser intensity was attenuated by neutral density filters such that the emitted photon counting rate was reduced to <3 kHz to avoid photon pile-up. The PM tube was a Hamamatsu R955, and the photon counting system had a response time of ~500 ps in FWHM. For lifetime measurements, the excitation polarizer was set at the vertical direction and the emission polarizer was oriented at the magic angle (54.7° from the horizontal). The emission was detected at a right angle to the excitation beam. The emission wavelengths were selected with a 4-nm band pass monochromator (Instrument SA, Inc.) or a Ditric 3-cavity 334-nm interference filter. Decay curves were collected into 1024 channels of a multichannel analyzer at a resolution of 20–30 ps/channel until 2×10^4 photons were collected in the peak channel. The decay data were fitted to a sum of exponential terms using a least-squares reconvolution procedure as previously described (Liao et al., 1992)

$$F(t)=\Sigma\alpha_i\exp(-t/\tau_i)$$

where τ_i are the lifetimes and α_i are the associated fractional amplitudes. The goodness-of-fit was evaluated by the reduced chi-squares ratio (χ^2_R), the weighted residuals, and the Durbin-Watson parameter (*D-W*). In the present experiments, $0.9 < \chi^2_R < 1.2$, and $D\text{-}W > 1.8$.

Reagents and chemicals

IAANS was obtained from Molecular Probes (Eugene, OR) and used without further purification. It was dissolved in dimethylformamide at concentrations of 10–15 mM and stored in the dark at either 4 or −20°C. The catalytic subunit of protein kinase A from beef heart was obtained from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

RESULTS

This study was carried out to investigate, under different ionic conditions, the fluorescence properties of IAANS attached to Cys-35 of cTnC(C84S) and Cys-84 of cTnC(C35S) reconstituted with cTnI and cTnT. These spectral properties were used to deduce the nature of conformational changes induced by Ca^{2+} binding to the regulatory domain. For comparison, the fluorescence properties of native cTnC doubly labeled with IAANS were also examined in parallel experiments.

Steady-state fluorescence

Fig. 1 shows the emission spectra of IAANS in doubly labeled native cTnC (*A*), singly labeled mutant at Cys-84 (*B*), and singly labeled mutant at Cys-35 (*C*). In the absence of divalent cations, the fluorescence intensity of IAANS attached to Cys-35 (Fig. 1 *C*, *curve 1*) was threefold lower than that of the probe attached to Cys-84 (Fig. 1 *B*, *curve 1*) or attached to both Cys-35 and Cys-84 (Fig. 1 *A*, *curve 1*). The spectral peaks of these three spectra were within 1–2 nm of each other (Table 1). In the presence of Mg^{2+}, the fluorescence intensity of each labeled protein decreased by 10–15% with no detectable spectral shift. When both Mg^{2+} and Ca^{2+} were present, the intensity of the probe attached to both Cys-35 and Cys-84 (Fig. 1 *A*) or to Cys-84 (Fig. 1 *B*) increased by a factor of ~2.0 and 1.6, respectively. These increases were accompanied by a blue spectral shift of 5 nm. These spectral changes are consistent with the observations reported previously (Johnson et al., 1980; Zhang et al., 1992; Dong et al., 1996). IAANS linked to Cys-35 responded to the addition of Ca^{2+} with little or no changes in intensity and spectral shift (Fig. 1 *C*). The fluorescence quantum yields and emission maxima determined for these and other reconstituted troponin systems are summarized in Table 1.

Fig. 2 shows a family of fluorescence emission spectra that were obtained from binary complexes of IAANS-labeled native cTnC and the two labeled cTnC mutants, each reconstituted with cTnI. For all three complexes, the intensity observed in the absence of divalent cations increased significantly when compared with that of the isolated labeled cTnC (Fig. 1). A 1.5-fold increase was observed for native cTnC with an 8-nm blue spectral-shift (*curve 1* of Fig. 2 *A* versus *curve 1* in Fig. 1 *A*). Similar changes were observed for the binary complex formed between the cTnC mutant labeled at Cys-84 and cTnI (Fig. 2 *B*, *curve 1*). With

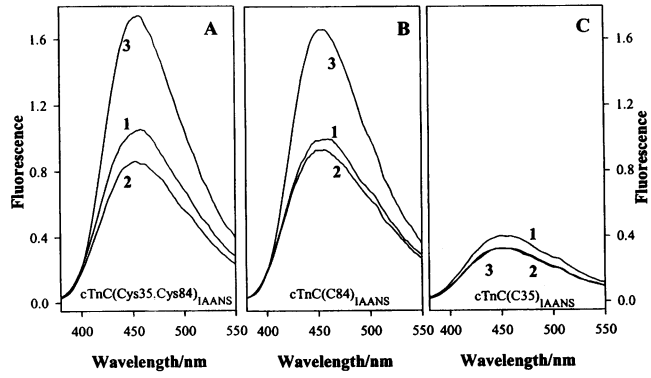

FIGURE 1 Fluorescence emission spectra of native cTnC labeled with IAANS at Cys-35 and Cys-84 (*A*), mutant cTnC(C35S) labeled at Cys-84 (*B*), and mutant cTnC(C84S) labeled at Cys-35 (*C*). Curve 1: EGTA; curve 2: Mg^{2+}; curve 3: Mg^{2+} + Ca^{2+}. The peak intensities of the spectra were normalized to the peak intensity of curve 1 (*B*). All samples contained 2 μM labeled protein, 2 mM EGTA, 0.3 M KCl, and 30 mM MOPS at pH 7.2, and were excited at 325 nm.

TABLE 1 Fluorescence properties of cTnC and cTnC mutants and their complexes with cTnI and cTnT

Sample	in EGTA			in Mg^{2+}			in Ca^{2+}		
	λ_{em} (nm)	Q_{rel}	$\langle\tau\rangle$ (ns)	λ_{em} (nm)	Q_{rel}	$\langle\tau\rangle$ (ns)	λ_{em} (nm)	Q_{rel}	$\langle\tau\rangle$ (ns)
$cTnC(Cys\text{-}84)_{IAANS}$	459	1.00	4.32	459	0.95	4.33	456	1.54	6.23
Binary complex	451	1.58	5.57	451	1.48	5.56	453	1.61	5.66
Ternary complex	451	1.63	5.64	451	1.56	5.42	455	1.56	5.52
p-binary complex	454	1.21	5.21	454	1.21	5.08	457	1.68	5.44
p-ternary complex	455	1.36	5.11	455	1.31	4.93	453	1.58	5.31
$cTnC(Cys\text{-}35)_{IAANS}$	460	0.35	4.06	460	0.29	4.10	461	0.29	4.01
Binary complex	449	1.00	5.24	450	0.88	4.95	458	0.3	3.61
Ternary complex	451	0.97	5.25	452	0.87	5.09	460	0.34	3.23
p-binary complex	449	0.94	5.08	452	0.88	5.00	458	0.55	4.14
p-ternary complex	449	0.91	5.09	451	0.92	5.19	457	0.59	4.28

$cTnC(Cys\text{-}84)_{IAANS}$ is a cTnC mutant labeled with IAANS at Cys-84, and $cTnC(Cys\text{-}35)_{IAANS}$ is cTnC mutant labeled with IAANS at Cys-35. The binary complex under each group of samples is the complex formed between the IAANS-labeled cTnC species in that group and native cTnI, and ternary complex is the complex formed with the labeled cTnC and native cTnI and cTnT. *p*-binary complex is the binary complex in which the cTnI is phosphorylated by PKA, and *p*-ternary complex is the complex in which the cTnI subunit is phosphorylated. The quantum yield of $cTnC(Cys\text{-}84)_{IAANS}$ was 0.086, and the relative quantum yields (Q_{rel}) of all samples were normalized to this value. Experimental errors of the quantum yield were <10% at all conditions. $\langle\tau\rangle$ is the intensity-weighted mean lifetime and is given by $\langle\tau\rangle = \Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$. The experimental errors of $\langle\tau\rangle$ were <6.0%. The experimental errors of the emission maximum were ±1 nm at all conditions. Measurements were made at 20°C in the basic buffer. EGTA concentration was 1 mM, Mg^{2+} concentration was 3 mM, and Ca^{2+} concentration was 2 mM.

the complex from the cTnC mutant singly labeled at Cys-35 (Fig. 2 *C*, *curve 1*), the increase in intensity was a factor of 3 with a 10-nm blue shift of the emission maximum. The considerably larger increase in the intensity of IAANS at Cys-35 of cTnC induced by complexation with cTnI would suggest a substantial alteration of the immediate environment of Cys-35 in the binary complex.

In the presence of Mg^{2+}, the intensity of the two binary complexes of cTnI with the labeled cTnC mutants decreased by 5–10% with no spectral shift (Fig. 2, *B* and *C*, *curve 2*). The presence of both Mg^{2+} and Ca^{2+} decreased the intensity of the complex obtained with doubly labeled native cTnC by 24% with a 5-nm red spectral shift (Fig. 2 *A*, *curve 3*), in agreement with early reports (Johnson et al., 1980: Robertson et al., 1982). This large Ca^{2+} effect was not observed with the binary complex formed between the cTnC mutant labeled at Cys-84 and cTnI (Fig. 2 *B*, *curves 2* and *3*). However, the cTnC mutant singly labeled at Cys-35 complexed to cTnI responded to Ca^{2+} with a 2.3-fold decrease in the IAANS intensity and a 9-nm red spectral shift (Fig. 2 *C*, *curve 3*). These results suggest that the fluorescence properties of IAANS linked to Cys-35 were largely responsible for the Ca^{2+}-induced spectral changes observed with native cTnC doubly labeled with IAANS at both Cys-35 and Cys-84. IAANS attached to Cys-35 of cTnC sensed only Ca^{2+}-induced environmental changes that occurred in the binary complex with cTnI. This is in contrast to the probe located at Cys-84, which was sensitive to Ca^{2+}-induced conformational changes in cTnC (Fig. 1 *B*), but not to changes that occurred in the binary complex. Similar fluorescence studies were also carried out with ternary complexes of IAANS-labeled native cTnC and the two IAANS-labeled cTnC mutants with cTnI and cTnT (data not shown). Essentially identical results were obtained as for the corresponding binary complexes. These results are summarized in Table 1.

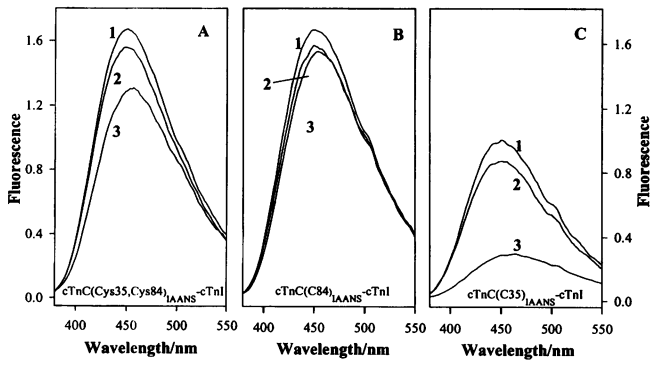

FIGURE 2 Fluorescence emission spectra of binary complexes of IAANS-labeled cTnC with native cTnI. (*A*) native cTnC doubly labeled at Cys-35 and Cys-84, (*B*) mutant cTnC(C35S) singly labeled at Cys-84, and (*C*) mutant cTnC(C84S) singly labeled at Cys-35. Curve 1: EGTA; curve 2: Mg^{2+}; curve 3: Mg^{2+} + Ca^{2+}. The peak intensities of the spectra were normalized to that of uncomplexed, mutant cTnC(C35S) labeled at Cys-84 determined in the presence of EGTA (Fig. 2 *B*, *curve 1*). This normalization makes possible direct comparison of the emission of all six samples shown in both Figs. 1 and 2. Samples contained 2 μM of labeled protein. Other conditions were the same as for Fig. 1.

Also listed in Table 1 are the steady-state fluorescence properties of the binary and ternary complexes of the three labeled species of cTnC reconstituted with cTnI phosphorylated by protein kinase A. In the absence of divalent cations or in the presence of Mg^{2+}, the phosphorylation of cTnI generally had little or no effect on the apparent spectral properties of the probe attached to both cysteine residues in the complexes. In the presence of Mg^{2+}, the probe at Cys-84 detected small environmental changes due to the cTnI phosphorylation in both the binary and ternary complexes. However, IAANS attached to Cys-35 appeared insensitive to the phosphorylation. In the presence of Ca^{2+},

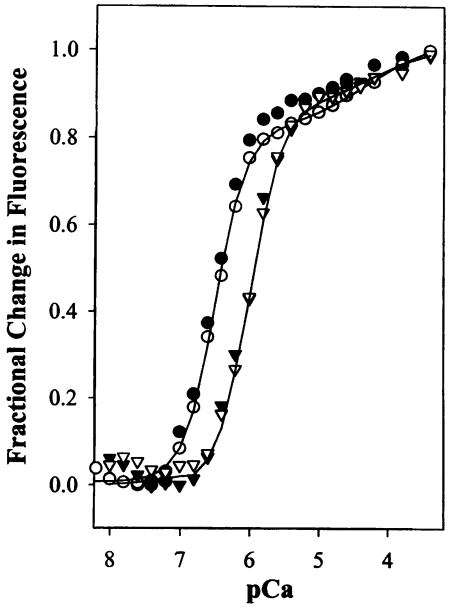

FIGURE 3 Ca^{2+} titration of mutant cTnC(C84S) singly labeled with IAANS at Cys-35 and complexed with native nonphosphorylated cTnI (*circles*) and phosphorylated cTnI (*triangles*). The titration was monitored by the change in the fluorescence of the probe (465 nm) with 325 nm excitation, and was carried out in the presence of EGTA (*open symbols*) and EGTA + Mg^{2+} (*closed symbols*). The fractional change in fluorescence is given by $(F - F_0)/(F_m - F_0)$, where F is the intensity observed at a given Ca^{2+} concentration, F_0 is the intensity in the absence of Ca^{2+}, and F_m is the intensity observed at a saturating level of Ca^{2+}.

the fluorescence of IAANS attached to both Cys-35 and Cys-84 increased by ~35% in the binary complex containing the phosphorylated cTnI, but the intensity was essentially unaltered in the ternary phosphorylated complex. This sensitivity observed with the binary phosphorylated complex appeared to be due to the probe at Cys-35, as the fluorescence of IAANS linked to Cys-35 increased by 40 and 70% in the binary and ternary complexes, respectively, after cTnI was phosphorylated.

Ca^{2+} titration of reconstituted troponin

A set of typical fluorescence titration curves of Ca^{2+} binding to the cTnC mutant labeled at Cys-35 and reconstituted with nonphosphorylated and phosphorylated cTnI are shown in Fig. 3. For the complex reconstituted with nonphosphorylated cTnI, the half-maximum increase in fluorescence change occurred at pCa 6.54, with a Hill coefficient of 1.5, corresponding to an apparent association constant of $3.47 \times 10^6\ M^{-1}$. This value decreased to $8.03 \times 10^5\ M^{-1}$ in the complex reconstituted with the phosphorylated cTnI, corresponding to pCa 5.91 with a Hill coefficient of 1.5. This decrease in the association constant of Ca^{2+} to cTnC is very similar to the values previously reported with cTnC doubly labeled at both Cys-84 and Cys-35, (Robertson et al., 1982), and indicates that IAANS attached at Cys-35 of cTnC can sense Ca^{2+} binding to the regulatory binding site II of cTnC in the binary complex. The apparent Ca^{2+} binding constants for different systems are summarized in Table 2.

Fluorescence intensity decay

The intensity decays of all IAANS-labeled cTnCs and their binary or ternary complexes with other troponin subunits followed a triexponential decay law with two major components, in the ranges of 2–3 and 0.5–1 ns, and a minor long component in the range of 6–7 ns (data not shown). The origin of these three lifetimes were not clear at the present time. The weighted mean lifetimes for the systems studied are summarized in Table 1. These mean lifetimes were used to calculate the bimolecular dynamic quenching constants.

Isolated native $cTnC_{IAANS}$ and mutant cTnC labeled at Cys-84 had mean lifetimes of 4.7 and 4.3 ns, respectively. Mg^{2+} had little effects on these mean lifetimes, but Ca^{2+} binding to the regulatory site increased the mean lifetimes to ~6 ns. This Ca^{2+}-induced increase in mean lifetime was due to conformational changes in the Cys-84 region and resulted from a significant reduction of nonradiative processes due to internal quenching in the apo state (Dong and Cheung, 1996). The observed mean lifetime of IAANS attached to Cys-35 was shorter (~4.0 ns) than that observed in the other two proteins, and was insensitive to the presence of either Mg^{2+} or Ca^{2+}. These properties are in accord with the steady-state spectral results.

Upon reconstitution with cTnI (binary complex) In the absence of divalent cations, the mean lifetimes of IAANS in native cTnC and the mutants increased by ~1 ns to 5.3–5.6 ns. No further changes were observed with fully reconstituted ternary complexes. The increase in lifetime observed

TABLE 2 Binding constants of IAANS-labeled cTnC and cTnC mutants and their complexes with cTnI and cTnT for Ca^{2+}

Sample	$K \times 10^{-5}$, M^{-1} in EGTA	$K \times 10^{-5}$, M^{-1} in Mg^{2+}
$cTnC(Cys\text{-}35,Cys\text{-}84)_{IAANS}$	4.3 ± 0.6 (1.4)	4.3 ± 0.6 (1.4)
Binary complex	27.8 ± 3.1 (1.5)	29.8 ± 3.5 (1.3)
Ternary complex	28.7 ± 3.0 (1.4)	26.7 ± 3.0 (1.4)
p-binary complex	9.0 ± 0.8 (1.3)	8.9 ± 0.9 (1.3)
p-ternary complex	8.8 ± 0.7 (1.4)	8.9 ± 0.8 (1.3)
$cTnC(Cys\text{-}84)_{IAANS}$	4.7 ± 0.5 (1.6)	6.0 ± 0.7 (1.7)
$cTnC(Cys\text{-}35)_{IAANS}$	—	—
Binary complex	34.7 ± 3.2 (1.5)	33.6 ± 3.4 (1.5)
Ternary complex	33.2 ± 3.6 (1.5)	32.0 ± 2.8 (1.6)
p-binary complex	8.0 ± 0.7 (1.3)	8.2 ± 0.5 (1.3)
p-ternary complex	8.1 ± 0.6 (1.3)	8.3 ± 0.8 (1.4)

Notations for samples are the same as given for Table 1. The binding constants were obtained from titration curves similar to those shown in Fig. 3, and the curves were analyzed by nonlinear least-squares fits as previously described (Dong et al., 1996). The numbers in parentheses are the Hill coefficient from the least-squares fits. The experimental errors for the Hill coefficient were <0.2. The measurements were made in the basic buffer. EGTA concentration was 1 mM, and Mg^{2+} concentration was 3 mM.

with apo binary complexes suggested that interaction between cTnC and cTnI altered the environment of the probe at both Cys-84 and Cys-35. Addition of Mg^{2+} or Ca^{2+} had little effect on the mean lifetime of IAANS attached to Cys-84. However, the presence of Mg^{2+} + Ca^{2+} decreased this mean lifetime to 3.3–3.6 ns for both the binary and ternary complexes reconstituted with cTnC labeled at Cys-35. This Ca^{2+}-induced change in the mean lifetime was consistent with the observed fluorescence quantum yield changes (Table 1).

Generally, the binary or ternary complexes of cTnC reconstituted with the phosphorylated cTnI increased the mean lifetime of IAANS, but the increase was not as much as that observed with nonphosphorylated cTnI. The lifetimes were insensitive to the presence of Mg^{2+}, but varied with the presence of Ca^{2+} (Table 1). Unlike the complexes reconstituted with nonphosphorylated cTnI, Ca^{2+} binding to the regulatory site II of the mutant labeled at Cys-84 reconstituted with the phosphorylated cTnI increased the mean lifetime by 8%, consistent with the steady-state data. Ca^{2+} binding to site II of the mutant labeled at Cys-35 reconstituted with phosphorylated cTnI decreased the mean lifetime by 5–10%, as compared with a 30–35% decrease observed with complexes reconstituted with nonphosphorylated cTnI (Table 1).

Fluorescence quenching

To estimate the accessibility of solvent to residue Cys-84 and Cys-35 in cTnC and to acquire information on the environment of IAANS attached to these two residues, the fluorescence of IAANS attached to these residues was studied in the presence of acrylamide. The Stern-Volmer plots for native cTnC and the two cTnC mutants, each labeled with IAANS, were essentially linear (data not shown), regardless of whether the labeled proteins were uncomplexed or complexed with the other subunits. Phosphorylation of cTnI did not affect the apparent linearity of the Stern-Volmer plots for the binary or ternary complexes. The bimolecular dynamic quenching constants (k_q) derived from the data are listed in Table 3. In the absence of added cations, the values of k_q for IAANS attached to Cys-84 of the cTnC mutant in the binary and ternary complexes were reduced by 12–15% when compared with the value for the uncomplexed cTnC. The phosphorylation of cTnI had no effect on k_q for the binary complex, but a small reduction (14%) for the ternary complex. The effect of added Mg^{2+} on the quenching constant was very small for both complexes, but the presence of both Mg^{2+} and Ca^{2+} substantially reduced k_q (33–24%) in the binary and ternary complexes. The phosphorylation of cTnI in these complexes further reduced k_q by 23–35%. These results would suggest considerable Ca^{2+}-induced shielding of IAANS linked to Cys-84 in the reconstituted apo-troponin. This shielding was further increased when the regulatory site of cTnC was saturated by Ca^{2+}.

The solvent accessibility of Cys-35-IAANS was substantially higher than that of Cys-84-IAANS, regardless of ionic conditions, indicating IAANS linked to Cys-35 to be more exposed than the same probe attached to Cys-84. The shielding of Cys-35-IAANS in the binary and ternary complexes, however, was considerably larger than Cys-84-IAANS in the same apo complexes or in the presence of Mg^{2+}. When the ionic condition was changed from Mg^{2+} to Mg^{2+} + Ca^{2+}, the value of k_q increased by a factor of 1.7 and 2.3 for the binary and ternary complexes, respectively. This large increased exposure was in contrast to the small increased exposure (1.10–1.16) observed for Cys-84-IAANS. Phosphorylation of cTnI generally increased k_q for the binary and ternary complexes in the absence or presence of divalent cations, although the combined effect of Mg^{2+} and Ca^{2+} was negligible.

TABLE 3 Fluorescence quenching constant for IAANS-labeled cTnC and cTnC mutants, and their complexes formed with cTnI and cTnT

Sample	$k_q \times 10^{-8}$, $M^{-1}s^{-1}$		
	in EGTA	in Mg^{2+}	in Ca^{2+}
$cTnC(Cys\text{-}84)_{IAANS}$	2.85 ± 0.31	3.27 ± 0.28	4.27 ± 0.37
Binary complex	2.51 ± 0.23	2.59 ± 0.28	2.84 ± 0.19
Ternary complex	2.43 ± 0.21	2.79 ± 0.32	3.25 ± 0.22
p-binary complex	2.51 ± 0.25	2.32 ± 0.18	2.21 ± 0.20
p-ternary complex	2.09 ± 0.18	2.27 ± 0.24	2.11 ± 0.18
$cTnC(Cys\text{-}35)_{IAANS}$	4.11 ± 0.33	4.17 ± 0.42	5.04 ± 0.47
Binary complex	1.60 ± 0.20	1.85 ± 0.23	3.08 ± 0.32
Ternary complex	1.49 ± 0.17	1.45 ± 0.11	3.28 ± 0.25
p-binary complex	1.98 ± 0.19	2.30 ± 0.17	3.29 ± 0.23
p-ternary complex	2.07 ± 0.14	2.54 ± 0.19	3.22 ± 0.29

See Table 1 for sample notations and conditions.

DISCUSSION

Cardiac muscle troponin C has two cysteine residues at positions 35 and 84. The sulfhydryl groups of these residues are readily alkylated by IAANS, and the doubly labeled cTnC has been extensively used to determine the Ca^{2+} sensitivity of myofibrillar ATPase and force development in skinned muscle fibers. Because the two residues are located in different regions within the regulatory N-domain, IAANS chemically linked to the two side chains must have different fluorescence properties. The fluorescence observed with the doubly labeled cTnC preparation must reflect the properties of two different local environments. This composite fluorescence cannot be used to follow Ca^{2+}-induced structural perturbations in the two regions. We have used two monocysteine cTnC mutants, each labeled with IAANS at its single sulfhydryl group, to examine changes in the local environments of individual cysteine residues without potential interference from the probe signal of the other residue, and to assess possible involvement of the two regions of the N-domain in subunit interactions in reconstituted cardiac muscle troponin.

The fluorescence properties of IAANS attached to Cys-84 in isolated cTnC mutant responds to the binding of Ca^{2+} to the single regulatory site with a large increase

(62%) in quantum yield (Q_{rel} from 0.92 to 1.49), a 3-nm blue spectral shift (459–456 nm), and a 2-ns increase in the mean lifetime (4.3–6.2 ns). Similar changes were previously observed with native cTnC selectively labeled with IAANS at Cys-84, and those changes have been interpreted in terms of a more "open" N-domain conformation in which internal quenching of IAANS-Cys-84 (located on helix D) by side chains surrounding Cys-84 is reduced (Dong and Cheung, 1996). Such an open conformation is consistent with computer models that predict Ca^{2+}-induced movements of helices B and C away from helices N, A, and D (Herzberg et al., 1986; Ovaska and Taskinen, 1991). These movements may lead to the exposure of a short hydrophobic stretch on helix B for Ca^{2+}-induced interaction with a target protein which, presumably, is troponin I. This interaction is believed to be the trigger of the contractile cycle. In the binary and ternary complexes reconstituted with cTnC mutant labeled at Cys-84 and the other troponin subunits, Q_{rel} remains relatively constant in the range of 1.4–1.6, and the mean lifetime is in the range of 5.4–5.7 ns, regardless of ionic conditions. The emission spectra of the complexes show a red-shift of 2–4 nm (451–455 nm) when the regulatory site is filled. These results indicate that a) the environment of Cys-84 in reconstituted apo-troponin may be very similar to that of Cys-84 in isolated cTnC saturated with Ca^{2+}, and b) the binding of Ca^{2+} to the regulatory site of cTnC in troponin does not significantly alter the Cys-84 environment. While it is not surprising that the Cys-84 region has different conformations dependent upon whether cTnC is isolated or in association with the other two troponin subunits, it should be noted that, even in the absence of bound activator Ca^{2+} at the regulatory site, the conformation of the Cys-84 region in the complexes is spectrally very similar to that of isolated cTnC, in which the regulatory site is occupied. It is not known to what extent the orientations of helices B and C in cardiac troponin may be different from the orientations in uncomplexed cTnC, but the orientations in the two states are likely different so as to bring about the observed enhancement of the radiative processes of IAANS attached to Cys-84 in the complexes. The present results raise the possibility that Ca^{2+}-induced rearrangements of the N-domain helices of cTnC in the troponin complex may be less extensive than as suggested by computer models on the basis of homologous EF-hand motifs of the C-domain and N-domain of the troponin C crystal structure.

The fluorescence properties of IAANS-Cys-35 in the cTnC mutant clearly indicate a highly exposed environment. These properties are little affected by Ca^{2+} binding to the regulatory site of the mutant, but are significantly changed when the mutant is reconstituted into apo-troponin. These fluorescence changes reflect the interaction between cTnC and cTnI and alteration of the local conformation of the Cys-35 region of the cTnC in troponin. These conformational changes result in a depressed nonradiative process of the probe. Upon binding Ca^{2+} at the regulatory site, this new conformation in the apo troponin appears to be reversed back toward that of isolated cTnC when the regulatory site is occupied by Ca^{2+}, as shown by an almost complete reversal of the quantum yield and a significant reduction in the lifetime. In this Ca^{2+}-loaded state of troponin, the fluorescence of Cys-35-IAANS is highly quenched when compared with that in the apo state. This quenching results from a combination of a) a more polar environment (9–10 nm red-shift), b) enhanced nonradiative processes (a 2.5-fold decrease in quantum yield and a 2.6-fold decrease in lifetime), and c) an increased exposure to solvent collision (a 2-fold increase in k_q). Cys-35 is located at the $-y$ coordinate of the 12-residue inactive Ca^{2+} binding loop of site I. IAANS attached to Cys-35 senses the Ca^{2+}-induced conformational change only in the presence of cTnI, but not when the cTnC is isolated, whereas the same probe attached to Cys-84 senses the conformational changes in the isolated cTnC. Thus, the inactive loop I may be involved in the Ca^{2+}-dependent cTnC-cTnI interaction. The present work with a monocysteine cTnC mutant labeled at Cys-35 has provided evidence that a segment of the inactive loop, if not the entire loop, responds to Ca^{2+} activation and strongly suggests participation of the inactive loop in the trigger mechanism. Using chimeric TnC in which the N-terminal 41 amino acids are of the cardiac type, Gulati et al. (1992) showed that cardiac-type site I and cardiac-type N-helix are essential for expression of the cardiac phenotype.

In a similar chimeric TnC in which the inactive Ca^{2+} binding loop I was activated to chelate Ca^{2+} by genetic modification, the cardiac-type Sr^{2+} sensitivity was found to transform into the skeletal-type (Gulati and Rao, 1994). Taken together, the present results and those of previous studies strongly suggest that flexible loop I, in which the side chains are not coordinated to a metal ion, may be essential as part of the trigger mechanism in cardiac muscle. Inasmuch as the helix-loop-helix segment of site I deviates from the typical EF-hand motif (Herzberg and James, 1988), and as a typical EF-hand motif is formed only upon chelating Ca^{2+}, it is also possible that an EF-hand at site I may prevent the N-terminal segment to participate in triggering the expression of the cardiac phenotype. The available information does not delineate these possibilities.

With monocysteine mutants of cTnC, we have used the environmental sensitive extrinsic fluorescent reagent IAANS to probe the local conformations of Cys-84 and Cys-35 of cTnC complexed with cTnI and reconstituted into the three-subunit cardiac troponin. Within the troponin complex, the Cys-84 region of helix D senses a relatively open conformation that may be similar to the open conformation of Ca^{2+}-loaded uncomplexed cTnC. Saturation of the single regulatory Ca^{2+} site does not appear to lead to a significant change in the Cys-84 environment. This is in contrast to Ca^{2+}-induced changes observed with isolated cTnC. Spectroscopic evidence has been obtained that shows involvement of the Cys-35 region of the native, inactive Ca^{2+} binding loop of site I in Ca^{2+} activation. This finding suggests that a flexible loop may dictate participation of cardiac-type inactive site I in the activation mechanism.

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