Ca²⁺- and Cross-Bridge-Dependent Changes in *N*- and *C*-Terminal Structure of Troponin C in Rat Cardiac Muscle

Donald A. Martyn,* Michael Regnier,* Daguang Xu,[†] and Albert M. Gordon[‡]

*Department of Bioengineering, University of Washington, Seattle, Washington 98195; [†]Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; and [‡]Departments of Physiology and Biophysics, University of Washington, Seattle, Washington 98195 USA

ABSTRACT　Linear dichroism of 5′-tetramethylrhodamine (5′ATR)-labeled cardiac troponin C (cTnC) was measured to monitor cTnC structure during Ca²⁺-activation of force in rat skinned myocardium. Mono-cysteine mutants allowed labeling at Cys-84 (cTnC(C84), near the D/E helix linker); Cys-35 (cTnC(C35), at nonfunctional site I); or near the *C*-terminus with a cysteine inserted at site 98 (cTnC-C35S,C84S,S98C, cTnC(C98)). With 5′ATR-labeled cTnC(C84) and cTnC(C98) dichroism increased with increasing [Ca²⁺], while rigor cross-bridges caused dichroism to increase more with 5′ATR-labeled cTnC(C84) than cTnC(C98). The pCa_{50} values and n_H from Hill analysis of the Ca²⁺-dependence of force and dichroism were 6.4 ($\pm$0.02) and 1.08 ($\pm$0.04) for force and 6.3 ($\pm$0.04) and 1.02 ($\pm$0.09) ($n = 5$) for dichroism in cTnC(C84) reconstituted trabeculae. Corresponding data from cTnC(C98) reconstituted trabeculae were 5.53 ($\pm$0.03) and 3.1 ($\pm$0.17) for force, and 5.39 ($\pm$0.03) and 1.87 ($\pm$0.17) ($n = 5$) for dichroism. The contribution of active cycling cross-bridges to changes in cTnC structure was determined by inhibition of force to 6% of pCa 4.0 controls with 1.0 mM sodium vanadate (Vi). With 5′ATR-labeled cTnC(C84) Vi caused both the pCa_{50} of dichroism and the maximum value at pCa 4.0 to decrease, while with 5′ATR-labeled cTnC(C98) the pCa_{50} of dichroism decreased with no change of dichroism at pCa 4.0. The dichroism of 5′ATR-labeled cTnC(C35) was insensitive to either Ca²⁺ or strong cross-bridges. These data suggest that both Ca²⁺ and cycling cross-bridges perturb the *N*-terminal structure of cTnC at Cys-84, while *C*-terminal structure is altered by site II Ca²⁺-binding, but not cross-bridges.

INTRODUCTION

In skeletal and cardiac muscle, the force-generating interaction between myosin and actin is inhibited by the troponin/tropomyosin complex on the thin filaments. The ability to generate force by myosin cross-bridges is regulated by myoplasmic [Ca²⁺]. Ca²⁺ binds to the thin filament regulatory protein, troponin C (TnC; one of three subunits of troponin), relieving the inhibition of myosin cross-bridge binding by the troponin/tropomyosin complex, and placing the thin filament in the "on state," thereby allowing strong cross-bridge binding and force generation (Lehrer, 1994; Tobacman, 1996; Gordon et al., 2000). Furthermore, there is significant structural (Vibert et al., 1997) and biochemical (McKillop et al., 1993; Geeves and Lehrer, 1994) evidence that, while Ca²⁺ binding to TnC allows the initial cross-bridge binding, subsequent strong cross-bridge interaction may be required for maximum availability of myosin binding sites on actin. Thus, strong cross-bridge binding may play an important role in thin filament activation, presumably by inducing structural changes in thin filament regulatory proteins that allow the thin filaments to more fully attain the "on" state for activation of contraction. In cardiac muscle the role cross-bridges play in facilitating thin filament activation may be even more dominant than in skeletal

muscle, as the Ca²⁺-affinity of cTnC is increased during force generation (Hofmann et al., 1987b; Pan and Solaro, 1987; Wang and Fuchs, 1994).

Although the gross myofilament structure of skeletal and cardiac muscle is similar, and the basic mechanisms of regulation of contraction by Ca²⁺ have similar molecular architectures, there are important differences in thin filament activation between skeletal and cardiac muscle (Gordon et al., 2000). In cardiac muscle cycling cross-bridge attachment significantly enhances the apparent affinity of TnC for Ca²⁺ and thin filament activation in cardiac muscle (Hofmann et al., 1987b; Pan and Solaro, 1987; Wang and Fuchs, 1994). By contrast, in skeletal muscle active cross-bridge binding neither enhances Ca²⁺ binding (Fuchs and Wang, 1991; Wang and Fuchs, 1994) nor induces structural changes in TnC (Martyn et al., 1999). These results imply that if cycling cross-bridge binding activates thin filaments in skeletal muscle, the structure of TnC is uncoupled from this process (Martyn et al., 1999). In contrast to cycling cross-bridges, the effects of rigor cross-bridge binding on thin filament activation are similar in skeletal and cardiac muscle. For example, rigor cross-bridges induce structural changes in sTnC (Zot and Potter, 1987; Allen et al., 1992; Martyn et al., 1999) and cTnC (Hannon et al., 1993; Putkey et al., 1997), and enhance thin filament activation (Brandt et al., 1990; Metzger, 1995) and Ca²⁺-binding (Bremel and Weber, 1972; Fuchs, 1977; Hofmann and Fuchs, 1987a; Pan and Solaro, 1987) in both tissues. These observations make it difficult to directly compare the effects of thin filament activation by cycling and rigor cross-bridges. Taken together, the evidence suggests that, while thin filament acti-

vation by rigor cross-bridges may occur by similar mechanisms, the role of strong, cycling cross-bridge binding in thin filament activation and regulation of contraction may be different in skeletal and cardiac muscle.

The goal of this study is to compare the effects on cTnC structure of Ca^{2+} binding to the single regulatory site (site II) on cardiac TnC (cTnC) with that of cycling, force-generating cross-bridges. Because, upon Ca^{2+} binding to site II, cTnC undergoes a complex series of interactions between the other subunits of the troponin complex, TnI and TnT (Farah and Reinach, 1995; Solaro and Rarick, 1998), we have used mono-cysteine mutants of cTnC to monitored the structure of cTnC at three sites; (1) at site I, in the N-terminus, with cTnC(C35); (2) near the junction of the D/E helixes, with cTnC(C84); and (3) at the C-terminus of cTnC, with insertion of a cysteine residue at site 98, near the structural Ca^{2+}/Mg^{2+} site III, with mono-cysteine mutant cTnC(C98). We used a "skinned" cardiac preparation, which enables us to control myoplasmic [Ca^{2+}] and exchange fluorescently labeled cTnC for endogenous cTnC. Ca^{2+}- and cross-bridge-dependent changes in cTnC structure were monitored by measuring the dichroism of 5′-tetramethyl-rhodamine (5′ATR), which was attached to the single cysteine residue in each cTnC mutant. The dichroism signal could indicate the mobility or order of the probe in its local environment on cTnC, the degree of order of the troponin complex, or the degree of order or orientation of the entire troponin/tropomyosin complex. In a complementary subset of experiments, cTnC(C84) structure was monitored with 2-(4′-iodoacetamidoanilo)naphthalene-6-sulfonic acid (IAANS), which is sensitive to the hydrophobicity or solvent accessibility of its environment. To allow separation of the effects of Ca^{2+}-binding and cross-bridge attachment on cTnC structure, cross-bridge attachment and force were inhibited with 1.0 mM sodium vanadate (Vi). Vi was chosen because inhibition of force and stiffness was nearly complete.

The results indicate that attachment of cycling cross-bridges is an important modulator of cTnC Ca^{2+} binding properties and cardiac thin filament activation. However, while the structure of cTnC is sensitive to both Ca^{2+} binding and cycling cross-bridge attachment, the responsiveness depends on the site of attachment of the fluorescent probe. The probe is sensitive to both Ca^{2+} and cross-bridges when attached near Ca^{2+}-regulatory site II, and only to Ca^{2+} binding to site II when attached at the C-terminus, and lacks sensitivity to either when attached near the nonfunctional site I of cTnC.

MATERIALS AND METHODS

Preparation and solutions

Male Sprague-Dawley rats (150–250 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and their hearts removed rapidly and placed in oxygenated Ringer's solution. Small trabeculae (100–200 μm diameter, 1–2 mm length) were dissected from the right ventricle and their surface membranes rendered permeable to bathing solution constituents by exposing them for at least 30 min at room temperature to a solution containing (in mM): 100 KCl, 9.0 MgCl$_2$, 4.0 MgATP, 5.0 K$_2$EGTA (ethylene glycol-bis-(b-aminoethylether)-N,N,N,N-tetraacetic acid), 10 MOPS (3-(n-morpholino) propane sulfonic acid), 1% non-ionic detergent Triton X100, pH 7.0, and 50% v/v glycerol. The skinned trabeculae were stored in the same solution without Triton X-100 at −20°C and used for experiments within 1 week. Experimental temperature was 20°C.

The composition of solutions was determined according to an iterative computer program that calculates the equilibrium concentration of ligands and ions based on published affinity constants. MOPS was used as the pH buffer and its concentration varied to maintain constant ionic strength. Ca^{2+} and K$^+$ were added primarily as the propionate salts. Rigor solutions contained no MgATP, creatine phosphate (CP), or creatine phosphokinase (CPK). All other solutions contained (in mM): EGTA 15; Mg^{2+} 1; MgATP 3; Na$^+$ 38; K$^+$ 95; CP 20; and 60 units/ml CPK.

Mechanical and fluorescence measurements

A detailed description of our optical and mechanical apparatus can be found in Martyn et al. (1999). The force transducer, length changer, and flow chamber for the skinned preparations are mounted on the moveable stage of an inverted epi-fluorescence microscope (Zeiss Axiovert 35). Initial sarcomere length was monitored by HeNe laser diffraction and set at 2.4 μm. In some experiments stiffness was measured by low-amplitude (0.2%) sinusoidal oscillations (500 Hz) of preparation length. Proprietary software was used to determine stiffness from Fourier analysis of the resulting force trace.

Quantitative epi-fluorescence measurement was made using a Zeiss Axiovert 35 microscope. The excitation light (HBO 50/3, OSRAM GmbH, Germany) was passed through an excitation filter, which is appropriate for the fluorophore being used and a 10× objective (Neofluor), which functioned as the condenser. The emitted light was collected by the same objective and passed through an appropriate band-pass filter to the photomultiplier (R928HA, Hamamatsu Photonics KK, Japan). Fluorescence (FL) and isometric force were recorded simultaneously on a chart recorder and stored digitally by computer. The background FL (that obtained without the fiber in the optical path) was subtracted from all measurements, and FL changes were expressed relative to that observed under relaxing conditions.

For excitation of 5′IATR-labeled TnC the fiber was illuminated by a mercury vapor lamp (HBO 50/3, OSRAM GmbH, Germany) filtered at 540 ± 5 nm, then passed through a 12% neutral density and polarizing filter oriented perpendicular to the fiber axis. The plane of polarization of the excitation illumination was alternated at 84 kHz by a photo-elastic modulator (Model PEM 80, Hinds Int'l., Portland, OR), with the peak-to-peak retardation set to 413 nm. The amount of light absorbed by the probes was determined by measuring the emitted light (590–630 nm), which will be focused onto a photomultiplier tube (Hamamatsu Type R938HA, Hamamatsu City, Japan). The output of the photomultiplier was monitored with a wide band-pass current to voltage converter (−3 db at 450 kHz). Because reflecting surfaces were present in both the excitation and emission optical paths, it was necessary to compensate each path for the preferential reflection of light polarized perpendicular to the fiber axis. This was accomplished with compensating windows consisting of two closely spaced glass plates. The windows could be angled so as to make the transmission of light polarized in either direction equal in both paths. To determine the rms amplitude of the sinusoidally varying difference in fluorescence intensity parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the fiber axis, the output of the amplifier was directed to a lock-in amplifier (Ithaco Model 3961B, Ithaca, NY). Dichroism or polarization anisotropy (r) were measured and expressed using the equation given by Tanner et al. (1992), as:

$$r = (-2D/L)/6J_2(\phi) + (1 - 3J_0(\phi)D/L)$$

where D is the rms value of $I_\parallel - I_\perp$ (from the output of the lock-in amplifier) and L is the total fluorescence ($I_\parallel + 2I_\perp$). The retardation of the photo-elastic modulator was set so that the zeroth order Bessel function (J_0) was zero. Further details are in Martyn et al. (1999).

The relation among force, fluorescence, and [Ca^{2+}] for each fiber were fitted by a nonlinear least-squares regression to the Hill equation, using SigmaPlot 5.0 (Jandel Corp., Chicago, IL):

$$\text{Relative force or fluorescence} = \frac{1}{1 + 10^{n_H}(pCa - pCa_{50})}$$

where pCa_{50} is the negative log of the [Ca^{2+}] that produces half-maximal force or fluorescence and n_H describes the slope of the Ca^{2+} dependence. The data from individual fibers were averaged and statistical analyses were performed using Excel (Windows 95, Microsoft Corp., Redmond, WA). Student's t-test was used to compare the means of data, with $p < 0.05$ considered significant.

cTnC extraction and reconstitution

Native cTnC was extracted from the skinned cardiac trabeculae by exposing them to a low ionic strength solution that contained (in mM): K_2EDTA 5; TRIS 20; and pH 7.2 (Gulati et al., 1991). To begin cTnC extraction the fiber was first placed in relaxing solution at 5–8°C, then rigor solution (zero [ATP]) at the low temperature. The length of the preparation was decreased to relieve rigor tension. The temperature was then raised to 30°C and the rigor solution was replaced with extraction solution at 30°C for 30–50 min. After cTnC extraction the procedure was reversed. Both the rigor and extraction solutions contained 5.0 mM BDM. After extraction the fiber was placed in relaxing solution at 20°C, and Ca^{2+}-activated force was determined in pCa 4.0 activating solution. Post-extraction force was 20 ± 5% (mean ± SEM; $n = 27$ preparations) of pre-extraction maximum values. Trabeculae were then reconstituted by incubation for 30 min in a relaxing solution (zero added Ca^{2+}), containing 10–50 μM fluorescently labeled cTnC. After reconstitution with fluorescently labeled mono-cysteine mutant, TnC force at pCa 4.0 was 80 ± 7.1% (mean ± SEM; $n = 24$ preparations) of pre-extraction maximum force.

Protein preparation and labeling

Preparation of mutant TnC's

Mono-cysteine mutants of cTnC that were made for specific placement of fluorescent probes are cTnC(C35S), cTnC(C84S), and cTnC(C35S,C84S,S98C). To facilitate easy identification of the fluorescent probe attachment, these mono-cysteine cTnC mutants are referred to as cTnC(C84), cTnC(C35), and cTnC(C98), respectively. The procedure for mutant cTnC preparation is also described in Dong et al. (1996).

A vector pET-24 (Novagen) containing the T7 promoter, lac operator, and a kanamycin resistance gene were used for the expression of cardiac TnC cDNA. The purified TnC cDNA digested with the restriction enzymes was ligated into the polylinker sites of pET-24. The recombinant DNA was then transformed into a cloning strain HMS 174 (Novagen). Transformed cells were grown on LB agar plated in the presence of kanamycin. Colonies were screened for inserts by digestion with the restriction enzymes. This procedure allowed us to check the construct insert before transforming recombinant plasmid into an expression host. The recombinant DNA (vector plus target gene) was subsequently transformed into host strain BL21(DE3) lysogen (Novagen) containing a gene induced by isopropyl-1-thio-b-D-galactopyranoside (IPTG) to synthesize T7 RNA polymerase. Transformed cells were grown on LB agar plates in the presence of kanamycin. A single colony was picked and inoculated into LB medium containing kanamycin at 37°C until the OD_{600} reached 0.8. IPTG was then added to a final concentration of 1 mM to induce the expression of target protein. The induced culture was incubated for at least 3 more hours and harvested by centrifugation. The cells were washed once with 50 mM Tris (pH 8.0), 2 mM EDTA, and then re-suspended in a solution containing 2.4 M sucrose, 1% Triton X-100, 50 mM Tris (pH 7.0), 10 mM EDTA, 1 mM DTT, and 0.5 mg/ml lysozyme. The cell suspension was placed on ice for 60 min and then sonicated for 5 min. The solution was centrifuged at 12,000 × g for 20 min at 4°C. An equal volume of 2× SDS sample buffer was added to an aliquot of sample from the supernatant for SDS gel analysis. The supernatant was exhaustively dialyzed against a buffer containing 6 M urea, 50 mM Tris (pH 8.0), 1 mM EGTA, 1 mM DTT before loading onto a DEAE-52 column equilibrated with the same urea buffer. Pure troponin C was eluted from the column with a linear gradient of 0–0.5 M KCl. TnC mutants were prepared by site-directed mutagenesis and their expression was done using T7-GEN In Vitro Mutagenesis Kit (United States Biochemical). The mutant proteins were expressed in the pET system, as mentioned above. The identity of the recombinant proteins was verified by DNA sequencing analysis. The purity of wild-type and mutant TnCs was determined on SDS-polyacrylamide gel electrophoresis.

5'ATR labeled cTnC

cTnC was dissolved in a buffer containing (in mM) 100 KCl, 20 TRIS, 1 ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) and pH 8.0. Ten mM DTT was added and the solution was cooled for 3–4 h. The protein was then dialyzed against the same buffer without DTT for 12 h with three solution changes to remove DTT. 5'IATR (20 mM in dimethylformamide) was added in 2× molar excess over TnC and the solution gently shaken at 10°C for 4 h. 5'ATR was kindly provided by John Corrie (Corrie and Craik, 1994). Finally, the labeled protein in buffer was dialyzed (3 times for 4 h apiece) against a buffer containing (in mM) 130 potassium propri-onate, 50 MOPS, 1 EGTA, pH 7.0.

IAANS-labeled cTnC

The mono-cysteine mutant cTnC-C35S was labeled with IAANS, as we previously described (Hannon et al., 1992). For fluorescence measurements skinned preparations with IAANS labeled cTnC were illuminated at 360 nm and the emitted light was filtered and collected at 520 ± 40 nm.

RESULTS

Force and fluorescence measurements

The Ca^{2+}-dependence of force and fluorescence were measured in detergent-skinned right ventricular trabeculae from rat hearts. Endogenous cTnC was extracted and the preparations were reconstituted with mono-cysteine mutants of cTnC, in which the single cysteine was labeled with 5'-ATR. The use of mono-cysteine mutants of cTnC allowed localization of the fluorescent probe at specific sites, enabling us to monitor localized Ca^{2+}- and cross-bridge-induced changes in cTnC structure. The average length and diameter of the skinned trabeculae used in this study were 1.75 ± 0.07 mm and 145 ± 9.8 μm (means ± SEM; $n = 24$), respectively. Initial sarcomere length was set at 2.4 μm. When it was possible to monitor sarcomere structure by laser diffraction during maximal activation, sarcomere length decreased to ~2.1 μm.

The experimental protocol is illustrated in Fig. 1 by representative traces of force (*top trace*) and fluorescence

FIGURE 1 Force (*top trace*) and fluorescence (*bottom trace*) changes at relaxing (pCa 9.2), and maximal (pCa 4.0) or submaximal (pCa 6.1) [Ca^{2+}] are shown for detergent-skinned right ventricular trabeculae from rat. The middle trace is the output from the lock-in amplifier, indicating the difference in emission with excitation parallel and perpendicular to the fiber axis ($I_{\|} - I_{\perp}$). The bottom trace represents total fluorescence ($I_{\|} + 2I_{\perp}$). The scale of the ($I_{\|} - I_{\perp}$) trace is 10 times that for total fluorescence. Changes in pCa (*arrows*) are indicated below the bottom trace. The shutters for the excitation lamp and photomultiplier tube were open for 2 s. The closed circle indicates background measurement, with the preparation out of the field of view. Experimental temperature was 20°C. The vertical calibration corresponds to 200 mN/mm^2 and the horizontal bar indicates 1 min.
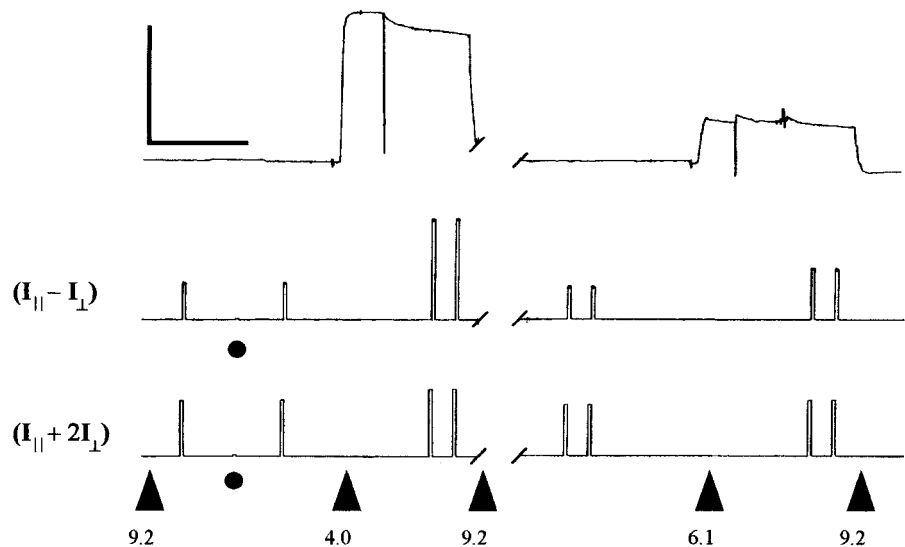
obtained under relaxed and maximal Ca^{2+}-activated conditions, and at a submaximal level of Ca^{2+}-activation (pCa 6.1), in a preparation that was reconstituted with 5'ATR-labeled cTnC(C84). The middle traces of Fig. 1 indicate the difference in 5'ATR emission parallel and perpendicular to the preparation axis, while the bottom fluorescent traces indicate the level of total fluorescence. Dichroism is expressed as the ratio of the difference trace to the total fluorescent intensity. Total fluorescence exhibited little dependence on Ca^{2+}, except for a small increase at pCa 4.0 (<5%), which was probably related to internal shortening of sarcomeres and the accompanying increase in the amount of fluorescent label in the field of view. By comparison, the difference signal (Fig. 1, *middle traces*) increased significantly, indicating an approximately twofold increase in dichroism. The increase in dichroism indicates either an increase in the order of the probe (or a decrease in probe mobility), or a change in average probe angle. During Ca^{2+} activation of force, dichroism increased in preparations that were reconstituted with either 5'ATR-labeled cTnC(C84) or cTnC(C98) (see Table 1).

Comparisons of the Ca^{2+}-sensitivity of force before extraction of native cTnC and reconstitution with either native cTnC or cTnC(C84) indicated that neither the extraction/

reconstitution protocol nor substitution of cTnC(C84) for native cTnC had a significant effect on force-pCa relations (data not shown). The similarity of Ca^{2+}-sensitivity of force for native and cTnC(C84) is consistent with previously published data that the substitution of serine for cysteine at site 35 has no significant effect on either the equilibrium binding or kinetics of Ca^{2+}-binding at site II of isolated IAANS-labeled cTnC (Dong et al., 1996; Hazard et al., 1998) or the maximum level of cardiac myofibrillar ATPase activity (Putkey et al., 1993). However, we found that extraction of endogenous cTnC and replacement with 5'ATR-labeled cTnC(C84) in skinned trabeculae caused a 0.14 ± 0.07 (mean ± SEM; n = 4 fibers) pCa unit increase of the pCa$_{50}$ of force and no significant change in n_{H}. This indicates that the 5'ATR probe caused some perturbation of cTnC structure when attached at Cys-84.

Effects of maximal Ca^{2+}-activation and strong cross-bridge binding on mono-cysteine mutants of cTnC

To compare the effects of Ca^{2+} and cycling and non-cycling cross-bridges on TnC structure, we measured force and

TABLE 1 Absolute dichroism (anisotropy) and relative changes in dichroism (mean ± SEM) resulting from activation with Ca^{2+} (pCa 4.0) and rigor cross-bridge binding, in the absence (9.2R) and presence (4.0R) saturating [Ca^{2+}]

Mutant cTnC	Number of Trabeculae	Dichroism (9.2)*	4.0[†]	9.2R[†]	4.0R[†]
cTnC(C84)	6	0.05 ± 0.002	1.86 ± 0.05	1.60 ± 0.05	1.78 ± 0.06
cTnC(C98)	5	0.03 ± 0.003	2.52 ± 0.16	1.21 ± 0.08	1.88 ± 0.12
cTnC(C35)	3	0.03 ± 0.005	0.93 ± 0.05	1.04 ± 0.04	1.03 ± 0.03

*Absolute value of dichroism at pCa 9.2.
[†]Expressed as a fraction dichroism obtained in relaxing solution (pCa 9.2).

corresponding changes in cTnC structure (dichroism) during thin filament activation by Ca^{2+} and rigor cross-bridge binding. The results are illustrated in Fig. 2, in which changes in force are expressed as a fraction of the maximum level at pCa 4.0 (after reconstitution with labeled cTnC) and changes in dichroism are expressed relative to values obtained at pCa 9.2. With the 5'ATR label attached to Cys-84 (cTnC(C84)), dichroism increased twofold upon maximal Ca^{2+}-activation of force (pCa 4.0) (Fig. 2 A). As shown in Fig. 2 A, rigor without Ca^{2+} (9.2R) caused dichroism to increase to ~65% of that found for Ca^{2+}-activation plus cycling cross-bridges (4.0). Addition of Ca^{2+} (4.0R) to trabeculae in rigor caused a further small increase in dichroism ($p < 0.05$). Thus, with the 5'ATR probe attached to Cys-84, the structure of cTnC appears to be altered by both Ca^{2+} and strong cross-bridge binding.

When the 5'ATR probe is attached to Cys-98 (cTnC(C98)), near Ca^{2+}/Mg^{2+} binding site III, dichroism increases ~2.5-fold during maximal Ca^{2+}-activated force in reconstituted trabeculae, as illustrated in Fig. 2 B (4.0). During activation by rigor cross-bridges alone (9.2R) dichroism increases slightly, but significantly ($p < 0.05$), above that found in relaxing solution (9.2). This small increase of dichroism with rigor indicates that strong cross-bridge attachment may have a small effect on cTnC structure in the C-terminus. These results contrast with the dichroism of 5'ATR attached at Cys-84, which increased substantially in rigor without Ca^{2+} (Fig. 2 A). Addition of Ca^{2+} to trabeculae in rigor caused an increase in dichroism of 5'ATR-labeled cTnC(C98) that was ~50% of the change at pCa 4.0 and active contraction (Fig. 2 B, Table 1). This result seems to indicate that rigor cross-bridges diminish the effect of Ca^{2+} binding on cTnC(C98) structure. Thus, when cTnC structure is monitored near site III by 5'ATR dichroism, changes in structure occur primarily in response to Ca^{2+} binding, with a smaller component due to cross-bridges, at least in rigor.

The 5'ATR-labeled mono-cysteine mutant cTnC(C35) was used to monitor the structural response of cTnC, near inactive site I, to Ca^{2+}-activation of force and the rigor cross-bridge state. The data are shown in Fig. 2 C. Unlike the observations with the fluorescent probe attached to either Cys-84 or Cys-98, the probe at Cys-35 did not indicate any significant change in cTnC structure during maximal Ca^{2+}-activated force or rigor with (4.0R) or without Ca^{2+} (9.2R). Thus, the structure of cTnC near site I, as measured by the orientation of 5'ATR at Cys-35, is insensitive to Ca^{2+} binding to site II and strong cross-bridge binding induced changes in thin filament activation. This observation contrasts with the report that rigor cross-bridge binding caused an increase in fluorescence in skinned cardiac muscle reconstituted with cTnC labeled at Cys-35 with IAANS, but agrees with the observations that Ca^{2+} binding and active force had no effect (Putkey et al., 1997). Because the 5'ATR probe was found to be relatively insensitive to experimental conditions when attached to Cys-35, the re-
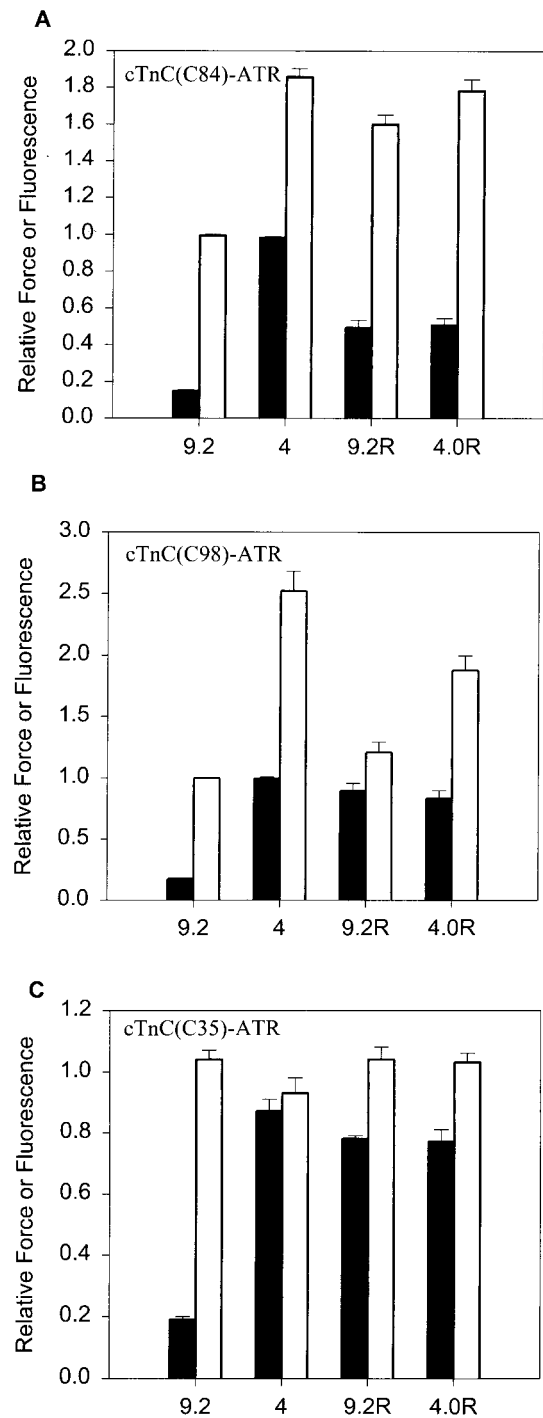
FIGURE 2 The effects of Ca^{2+}-activation of force and thin filament activation by rigor cross-bridge binding are illustrated for mono-cysteine mutants cTnC(C84) in (A), cTnC(C98) in (B), and cTnC(C35) in (C). Force and dichroism were determined at pCa 9.2 (9.2), pCa 4.0 (4.0), rigor without Ca^{2+} (9.2R), and rigor with Ca^{2+} (4.0R). The values of dichroism (*open bars*) are expressed as a fraction of the value obtained in relaxing solution (pCa 9.2), and force (*filled bars*) is expressed as a fraction of the value obtained upon maximal Ca^{2+}-activation (pCa 4.0).

mainder of the study was focused on the mono-cysteine mutants cTnC(C84) and cTnC(C98). The changes in force and dichroism from Fig. 2 are summarized in Table 1, along with the absolute values of dichroism (anisotropy) at pCa 9.2 for each 5'ATR-labeled mono-cysteine mutant.

The Ca²⁺-dependence of force and dichroism and the effects of force inhibition on 5'ATR-cTnC(C84) reconstituted trabeculae

The Ca²⁺-dependence of force and corresponding changes in cTnC structure were measured in skinned cardiac trabeculae reconstituted with 5'ATR-labeled cTnC(C84) (Fig. 3). Force and dichroism were determined at each [Ca²⁺] as described in Fig. 1, with particular care taken to bracket measurements made at submaximal Ca²⁺-activation. Force-Ca²⁺ and dichroism-Ca²⁺ data from each skinned trabeculae were fit by the Hill equation, and the derived Hill fit parameters are summarized in Table 2. The pCa_{50} of dichroism was slightly greater than force, while the slopes (n_H) of force and dichroism are not significantly different. Maximum Ca²⁺-activated force in cTnC(C84) reconstituted trabeculae was $97 \pm 1.6\%$ of pre-extraction controls (means $\pm$ SEM; $n = 5$ trabeculae).

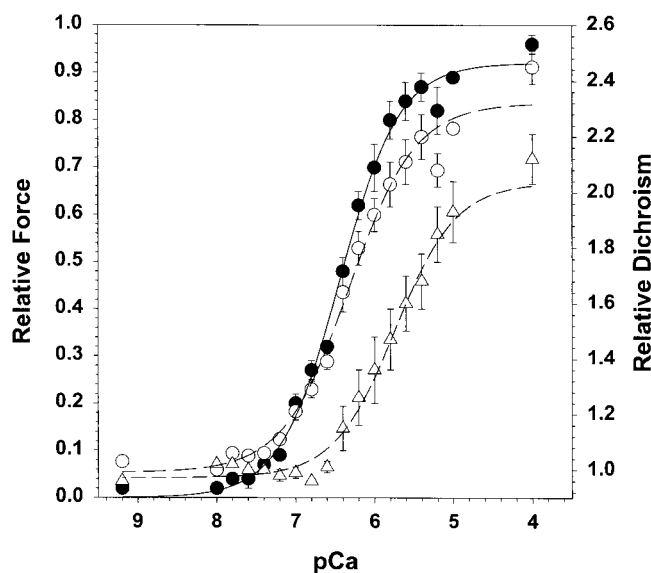Previous fluorescence studies of the effects of Ca²⁺ or strong cross-bridge binding on cTnC structure at Cys-84 used IAANS attached to native cTnC under conditions chosen to favor labeling at Cys-84 (Hannon et al., 1992) or with mono-cysteine cTnC(C84) (Putkey et al., 1997). To enable comparison with dichroism data (Fig. 3), we also determined the Ca²⁺-dependence of force and fluorescence in cTnC(C84)-IAANS-reconstituted skinned trabeculae. In these preparations the pCa_{50} of IAANS fluorescence was slightly more sensitive to Ca²⁺ than was force (Table 2), which is consistent with our previous finding (Hannon et al., 1992). However, this differs somewhat from the conclusions of Putkey et al. (1997), that the Ca²⁺-dependence of force and IAANS fluorescence are similar with IAANS-labeled cTnC(C84). Differences between the Ca²⁺-sensitivities of force and dichroism we observe for cTnC(C84) labeled with IAANS or 5'ATR (Table 2) could be due to internal sarcomere shortening during tension development that results from significant end-compliances in skinned cardiac muscle. In the case of IAANS-labeled cTnC, increasing internal shortening at higher forces could bring more fluorophore into view, resulting in a component of fluorescence that is related only to sarcomere length and not cTnC structure, and apparent greater sensitivity of fluorescence to Ca²⁺ than force. This would not be the case for 5'ATR dichroism because the polarized component of fluorescence is expressed as a fraction of the total fluorescence.

To test for the effects of cycling cross-bridge binding on the structure of 5'ATR-labeled cTnC(C84), the Ca²⁺-dependence of dichroism (Fig. 3) was measured when force was inhibited by 1.0 mM Vi (Figure 3, *open triangle*). Vi is a phosphate analog that binds to myosin S1 (Smith and Rayment, 1995) and is known to be a potent inhibitor of force in both skinned skeletal (Dantzig and Goldman, 1985; Martyn et al., 1993) and cardiac muscle (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994, 1995). At higher concentrations (10 mM), Vi treatment of skinned cardiac muscle results in extraction of troponin and generation of Ca²⁺-insensitive force (Strauss et al., 1992). However, we observed no elevation of resting tension or Ca²⁺-insensitive force in any experiment in which force was inhibited by 1.0 mM Vi, suggesting that no extraction of troponin had occurred. At pCa 4.0 in the presence of 1.0 mM Vi, force was $6.0 \pm 1.0\%$ and stiffness $10.0 \pm 3.0\%$ (mean $\pm$ SEM; $n = 7$ trabeculae) of the values obtained at pCa 4.0 without Vi. For 5'ATR-cTnC(C84) force inhibition resulted in a large decrease in the pCa_{50} of dichroism and a significant ($p > 0.05$) decrease in dichroism at pCa 4.0 (Fig. 3; Table 2). It is unlikely that the decreased maximum value and Ca²⁺-dependence of dichroism were the result of a direct effect of Vi on cTnC structure because neither the dichroism at pCa 9.2 nor the total fluorescence of either 5'ATR-labeled cTnC(C84) or cTnC(C98) reconstituted trabeculae was altered by 1.0 mM Vi (data not shown). This is supported by the findings that inorganic phosphate (of which Vi is an analog) does not alter Ca²⁺-binding to cTnC (Kentish and Palmer, 1989) and Vi decreased Ca²⁺-binding to cTnC in cardiac

FIGURE 3 The Ca²⁺-dependence of active force (●) and dichroism (○) are shown for skinned trabeculae reconstituted with 5'ATR-labeled cTnC(C84). The Ca²⁺-dependence of dichroism is also shown following inhibition of force with 1.0 mM Vi in the bathing solutions (△). Data (mean $\pm$ SEM) were obtained from five fibers. Both force (*solid curve*) and dichroism without (*dashed lines*) and with Vi (*short dashed lines*), data were fit with the Hill equation. The pCa_{50} values and slopes (n) of the fits are included in Table 2.

TABLE 2 The calcium-dependence of force and accompanying changes in cTnC structure monitored by changes in the dichroism of 5′ATR to either Cys-84 or Cys-98, or by changes in steady-state fluorescence of IAANS attached to Cys-84

cTnC	Probe	[Vi]	Measurement	No.	pK ($\pm$SEM)	n_H ($\pm$SEM)	% Max[#]
cTnC(C84)	ATR	0	Force	5	6.4 $\pm$ 0.02	1.1 $\pm$ 0.04	1.0
		0	Dichroism		6.3 $\pm$ 0.04	1.02 $\pm$ 0.09	1.0
		1.0	Dichroism	5	5.66 $\pm$ 0.05	1.1 $\pm$ 0.10	0.71 $\pm$ 0.03
cTnC(C98)	ATR	0	Force	5	5.53 $\pm$ 0.04	3.1 $\pm$ 0.64	1.0
		0	Dichroism		5.39 $\pm$ 0.03	1.87 $\pm$ 0.17	1.0
		1.0	Dichroism		5.15 $\pm$ 0.02	2.17 $\pm$ 0.17	0.93 $\pm$ 0.01
cTnC(C84)	IAANS	0	Force	4	5.56 $\pm$ 0.05	1.33 $\pm$ 0.15	
		0	Fluorescence		5.86 $\pm$ 0.23	0.93 $\pm$ 0.42	

[#]Expressed as a fraction of the increase in dichroism at pCa 4.0 without 1.0 mM Vi.

muscle, but not the cTnC isoform in slow skeletal fibers (Wang and Fuchs, 1994). Therefore, the observation that force inhibition decreases both the maximum magnitude and Ca^{2+}-sensitivity of dichroism (Fig. 3; Table 2), indicate that when monitored at Cys-84 the structure of cTnC is sensitive to both Ca^{2+}-binding to site II and strong, cycling cross-bridge attachment.

The Ca^{2+}-dependence of force and dichroism and the effects of force inhibition on 5′ATR-cTnC(C98) reconstituted trabeculae

In skinned trabeculae reconstituted with 5′ATR-labeled cTnC(C98), force is slightly more sensitive to Ca^{2+} than dichroism (Fig. 4; Table 2). Furthermore, the Ca^{2+}-dependence of force and dichroism is shifted to higher $[Ca^{2+}]$ and has steeper slopes than in cTnC(C84)-reconstituted preparations (Fig. 3; Table 2). Because unlabeled cTnC(C98) caused the same shift in force-Ca^{2+} relations (data not shown), the decrease in the Ca^{2+}-sensitivity of force with cTnC(C98) is likely due to the mutation of Ser-98 to Cys, and not to the 5′ATR probe. Maximum Ca^{2+}-activated force in cTnC(C98) reconstituted trabeculae was 87 $\pm$ 5.2% of pre-extraction controls (mean $\pm$ SEM; $n = 5$ trabeculae).

The effects of force inhibition by 1.0 mM Vi on the Ca^{2+}-dependence of dichroism was also measured in skinned trabeculae reconstituted with 5′ATR-labeled cTnC(C98) (Fig. 4, *open triangle*). Force inhibition with 1.0 mM Vi caused a decrease in the Ca^{2+}-sensitivity of dichroism that was less than the decrease observed with cTnC(C84) (Fig. 3) and no change in slope of dichroism-Ca^{2+} relations. Also, force inhibition with Vi caused no significant decrease in dichroism at pCa 4.0 ($p < 0.05$), in contrast to the results with cTnC(C84) (Fig. 3). The Hill fit parameters for the data in Fig. 4 are included in Table 2. Thus, in contrast to cTnC(C84) (Fig. 3; Table 2) force inhibition causes a smaller decrease in the Ca^{2+}-sensitivity of structural changes in the *C*-terminus of cTnC and no change in cTnC(C98) structure at saturating $[Ca^{2+}]$.

The data in Figs. 2 *B* and 4 indicate that in cTnC with Cys engineered at site 98, the label responded mostly to Ca^{2+} binding to regulatory site II, while the data in Figs. 2 *A* and

3 indicate that at Cys-84 the label responded to both Ca^{2+} and cross-bridge binding. Experiments described in Figs. 3 and 4 were done under identical conditions. Data from both 5′ATR-labeled mono-cysteine cTnC mutants suggest that force inhibition with Vi results in a decrease in the apparent affinity of cTnC for Ca^{2+}, and supports previous observations that force inhibition in cardiac muscle caused decreased thin-filament Ca^{2+} binding (Hofmann and Fuchs, 1987b; Wang and Fuchs, 1994).

DISCUSSION

The results of this study are in accord with measurements of Ca^{2+}-binding in skinned cardiac muscle and support the idea that the structure of cTnC is further modified and its
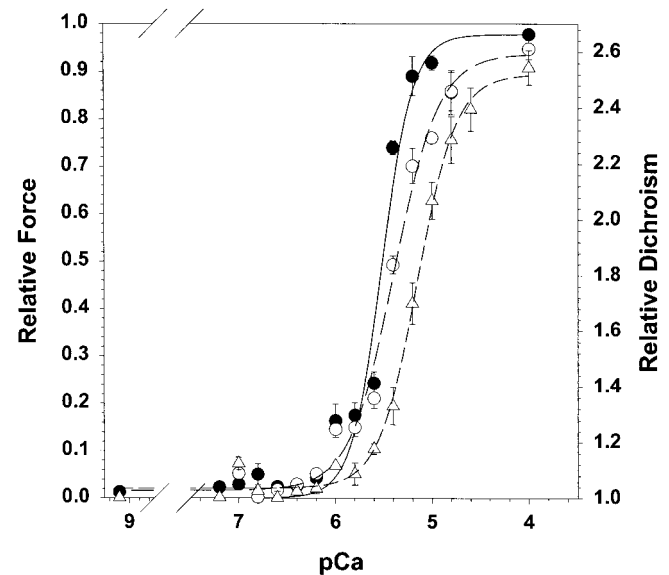
FIGURE 4 The Ca^{2+}-dependence of force (●) and dichroism (○) were determined for 5′ATR-labeled cTnC(C98) reconstituted trabeculae. The Ca^{2+}-dependence of dichroism (△) following force inhibition with 1.0 mM Vi is also shown. Values are the mean ($\pm$SEM) for data obtained from four skinned trabeculae. Data for force (*solid line*) and dichroism without (*dashed line*) and with (*short dashed line*) Vi were fit by the Hill equation, and the corresponding Hill parameters are included in Table 2.

Ca^{2+}-affinity enhanced by strongly bound, cycling cross-bridges (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994). Furthermore, we find that when measured by dichroism of 5′ATR attached to cTnC, the structural response of cTnC to Ca^{2+} and cross-bridges depends on the site of labeling, as illustrated in Figs. 2–4. Our results are complementary to those previously obtained with IAANS-labeled native (Hannon et al., 1992) and mono-cysteine mutants of cTnC (Putkey et al., 1997), and extend those observations to include structural changes monitored at the *C*-terminus of cTnC in skinned cardiac trabeculae. Additionally, because measurement of 5′ATR dichroism potentially provides information about the degree of order or mobility of cTnC domains or probe orientation, the data complement those obtained with fluorescent probes that respond to local changes in solvent accessibility or hydrophobicity.

Structural responses of cTnC domains to maximal Ca²⁺-activation of force and rigor cross-bridges

The effects of Ca^{2+} or strong cross-bridge binding on the dichroism of 5′ATR were different with each mono-cysteine mutant cTnC. With the 5′ATR probe at Cys-84, rigor cross-bridge binding (without Ca^{2+}; 9.2R) causes an increase in dichroism that is nearly as large as the increase with maximal [Ca^{2+}] and cycling cross-bridges (Fig. 2 *A*; Table 1). Furthermore, saturating Ca^{2+} in rigor (4.0R; Fig. 2 *A*; Table 1) caused only a small increase in dichroism above pCa 4.0 controls, implying that the *N*-terminal structure of cTnC was perturbed to nearly the same extent by either maximal Ca^{2+}-binding to site II and cycling cross-bridges, or rigor cross-bridges alone. In contrast, rigor cross-bridges induced only a small change at Cys-98 (Fig. 2 *B*; Table 1), suggesting that the *C*-terminal structure of cTnC was more responsive to Ca^{2+}-binding to site II and relatively insensitive to strong cross-bridge binding. Probes attached at both sites responded well to Ca^{2+} binding (presumably to regulatory site II), implying that Ca^{2+}-binding to site II induced structure changes in both the *N*- and *C*-terminal domains, as for skeletal sTnC (Allen et al., 1992).

With the probe at Cys-98, addition of Ca^{2+} in rigor caused an increase of dichroism that is less than found for maximum Ca^{2+}-activation of force (4.0R; Fig. 2 *B*). This indicates that rigor cross-bridge attachment may reduce the responsiveness of cTnC structure at engineered Cys-98 to Ca^{2+}, even though the probe at the *C*-terminus was not perturbed by rigor cross-bridges alone. With rigor cross-bridge binding *N*-terminal structure would be significantly perturbed (9.2R; Fig. 2 *A*), so that addition of saturating Ca^{2+} in rigor could induce less of a change in *C*-terminal structure. However, the apparent differential sensitivity of *N*- and *C*-terminal domains of cTnC to Ca^{2+} and rigor cross-bridge binding is difficult to explain if Ca^{2+}-binding

to site II and strong cross-bridge attachment induce exactly the same structural changes in cTnC. If this were the case, the response of probes attached to either domain should respond to both Ca^{2+} and strong cross-bridge binding.

The absence of an effect of Ca^{2+} or strongly bound cross-bridges on the dichroism of 5′ATR-labeled cTnC(C35) reconstituted trabeculae (Fig. 2 *C*) indicates that the mobility of 5′ATR at Cys-35 in the nonfunctional site I of cTnC was not perturbed during activation of force. This result is different from that reported by Putkey et al. (1997) in which IAANS-labeled cTnC structure was altered by rigor, but not cycling cross-bridges. Also, activation-dependent changes in structure near site I could occur because the *N*-terminus of cTnC assumes a more "open" configuration, upon Ca^{2+}-binding to site II in the presence of cTnI (Dong et al., 1999) or cTnI fragments (Li et al., 1999). However, our results are not necessarily contradictory because local changes in hydrophobicity resulting from Ca^{2+}-binding to site II or TnI binding to the hydrophobic patch of cTnC (Sia et al., 1997; Dong et al., 1999) could occur without corresponding changes in 5′ATR mobility at Cys-35. However, because 5′ATR dichroism at Cys-35 was not altered by either Ca^{2+} or strong cross-bridge binding (Fig. 2 *C*; Table 1), changes in dichroism at labeled Cys-84 or 98 likely reflect changes in local structure and not an overall change in Tn orientation.

The response of 5′ATR-labeled cTnC to Ca²⁺ and cycling cross-bridges depends on the site of labeling

The Ca^{2+}-dependence of changes dichroism measured with 5′ATR attached to either Cys-84 (Fig. 3) or Cys-98 (Fig. 4) indicates that changes in cTnC structure occur at slightly higher [Ca^{2+}] than corresponding changes in active force. These results contrast with those obtained when cTnC structure is monitored with IAANS attached to Cys-84, in which fluorescence is either more sensitive to Ca^{2+} than force (Table 2) and Hannon et al. (1992) or have a similar Ca^{2+}-dependence (Putkey et al., 1997). These observations are important, because structural changes in cTnC that occur at or near Cys-84 are likely to be central to thin-filament activation, because this region of cTnC binds tightly to the inhibitory *C*-terminal region of cTnI in the presence of Ca^{2+} (Farah and Reinach, 1995; Solaro and Rarick, 1998). For example, we found a small increase in the Ca^{2+}-sensitivity of force resulting from attachment of the 5′ATR probe at C84, further indicating that perturbation of structure near this critical region can cause altered function. Also, changes in cTnC structure from Ca^{2+}-binding to site II or strong cross-bridge attachment may be reported differently by environmental probes that respond to altered hydrophobicity of the local environment, such as IAANS, and by 5′ATR, which provides information about the mobility of the probe in its local environment. Additionally, differences between

results with 5′ATR and IAANS could also arise from the relative sensitivity of IAANS fluorescence to sarcomere shortening during force generation in skinned cardiac muscle.

Inhibition of strong cross-bridge binding and force with 1.0 mM Vi enabled us to separate the effects of Ca^{2+}-binding on cTnC structure from those induced by strong, cycling cross-bridges. With 5′ATR attached to Cys-84 the probe responded to both Ca^{2+} and strong, cycling cross-bridge binding, with force inhibition causing a decrease in both Ca^{2+}-sensitivity and maximum magnitude of dichroism (Figs. 2 and 3; Table 2). This result is different from that reported by Putkey et al. (1997), where force inhibition with Vi caused no change in the Ca^{2+}-dependence or magnitude of fluorescence from skinned trabeculae containing IAANS-labeled mono-cysteine mutant cTnC(C84). However, the data in Fig. 3 are consistent with the decrease of Ca^{2+} bound to cardiac thin filaments under similar conditions (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994). Furthermore, the decrease in dichroism at pCa 4.0 following force inhibition indicates that in the absence of cross-bridge binding, saturating $[Ca^{2+}]$ does not fully perturb cTnC structure, which requires strong cross-bridge binding. This is consistent with the observation that the *N*-terminal domain of cTnC is less perturbed by Ca^{2+}-binding than sTnC (Sia et al., 1997; Spyracopoulos et al., 1997), and that maximal Ca^{2+}-binding to cTnC results in a distribution of cTnC between unperturbed and perturbed states (Dong et al., 1996, 1997). The decrease of dichroism at pCa 4.0 (Fig. 3) and the enhancement dichroism by rigor cross-bridges of 5′ATR-labeled cTnC(C84) (Fig. 2 *A*) support the idea that the *N*-terminal structure of cTnC is sensitive to both Ca^{2+} and strong cross-bridge binding. Thus the results argue that strong cross-bridge attachment results in additional activation of cardiac thin filaments, inducing structural changes in cTnC, even at saturating $[Ca^{2+}]$.

In contrast to cTnC(C84), 5′ATR-labeled cTnC(C98) exhibited a smaller decrease in the Ca^{2+}-sensitivity of dichroism and no significant ($p > 0.05$) decrease in dichroism at pCa 4.0 following force inhibition (Fig. 4; Table 2). It should be noted that while maximum Ca^{2+}-activated force was similar for cTnC(C84) and cTnC(C98) reconstituted trabeculae, substitution of cysteine for serine at site 98 did cause a significant decrease in the Ca^{2+}-sensitivity of force and dichroism (Fig. 4; Table 2), compared to 5′ATR-labeled cTnC(C84). This implies that cTnC(C98) structure was functionally altered from the native conformation. With the probe at Cys-98, changes in *C*-terminal structure could potentially occur in response to either Ca^{2+}-binding to the *N*-terminus, as proposed for skeletal TnC (Allen et al., 1992), or to Ca^{2+}/Mg^{2+} exchange at *C*-terminal sites III and IV (Johnson et al., 1980). However, the affinities of sites III and IV for either Ca^{2+} or Mg^{2+} are much higher than site II (Johnson et al., 1980), thus structural changes resulting from exchange at those sites should occur at lower $[Ca^{2+}]$.

5′ATR-cTnC(C98) dichroism-Ca^{2+} relations exhibited no evidence of an additional transition that could be attributed to changes in dichroism resulting from exchange at site III and IV, even with a shift to higher $[Ca^{2+}]$ following force inhibition (Fig. 4). Thus, unless Ca^{2+}/Mg^{2+} exchange in the *C*-terminus is influenced by cross-bridge binding, the Ca^{2+}-induced changes in 5′ATR-cTnC(C98) dichroism result from Ca^{2+}-binding to regulatory site II. Therefore, whether monitored by structural changes at the *C*- or *N*-terminus of cTnC, force inhibition in cardiac muscle decreases the apparent Ca^{2+}-affinity of site II, as reported from Ca^{2+}-binding experiments (Hofmann and Fuchs, 1987a,b; Wang and Fuchs, 1994). These results contrast with similar experiments in skinned fast skeletal fibers using sTnC labeled at Cys-98 with 5′ATR (Martyn et al., 1999). Force inhibition did not significantly change either the Ca^{2+}-sensitivity or magnitude of dichroism, indicating that the probe responded primarily to Ca^{2+}-binding at the *N*-terminus of sTnC and that cycling cross-bridges did not induce significant structural changes in sTnC at any level of activation (Martyn et al., 1999).

How can *C*- and *N*-terminal domains have different responses to Ca^{2+} and strong cross-bridge binding?

Our results indicate that in cardiac muscle, Ca^{2+} and cycling cross-bridge binding to cardiac thin filaments may induce different structural changes in the *C*- and *N*-terminus of cTnC. This differential responsiveness of cTnC domains could reflect differences in domain interactions between cTnC and cTnI, or even with cTnT. This might not be unexpected, because Ca^{2+}-binding to site II on cTnC causes thin-filament activation by increasing the affinity of cTnC for cTnI, effectively relieving acto-myosin inhibition by competing with actin for the inhibitory domains of cTnI (Farah and Reinach, 1995; Tobacman, 1996; Solaro and Rarick, 1998). However, cross-bridge binding could directly displace Tm on the surface of myosin and thus alter cTnC structure by altering its interactions with cTnI or cTnT. As suggested above, the sensitivity of *C*-terminal structure to Ca^{2+}-binding to site II, but not strong cross-bridge binding, is difficult to explain unless Ca^{2+}-binding to site II on cTnC and cross-bridge binding to thin filaments induce different structural states in cTnC. Recent observations indicate that different regions of skeletal TnI bind to the *N*- or *C*-terminal domains of skeletal TnC in a Ca^{2+}-dependent manner (Tripet et al., 1997), and thus offer a possible explanation for this differential sensitivity. The *C*-terminus of skeletal TnI has two actin binding sites, one (residues 96–115) that binds to the *C*-terminus of TnC, and another (residues 140–148) that binds to the *N*-terminus of TnC; an intervening sequence binds to the hydrophobic patch of TnC in the presence of Ca^{2+} (Tripet et al., 1997). Equivalent TnC binding sequences on cTnI (Leszyk et al.,

1988) and skeletal TnI (Wilkinson and Grand, 1975; Sheng et al., 1992) are nearly identical. If the *N*- and *C*-terminal domains of cTnC bind different regions of cTnI during Ca^{2+}-activation, then the effects of Ca^{2+} monitored at the *C*-terminus of cTnC could indicate only local changes in *C*-terminal, and not necessarily *N*-terminal, structure. Furthermore, if the two domains do not act in concert the *N*-terminus could be perturbed by Ca^{2+} and cross-bridges, and the *C*-terminus respond only to Ca^{2+}, as we observe. Thus, although both Ca^{2+} binding to cTnC and cycling cross-bridge interaction lead to thin-filament activation in cardiac muscle, presumably by shifting the position of tropomyosin to the "on" state (McKillop and Geeves, 1993), cross-bridge binding appears to alter the Ca^{2+}-binding properties of cardiac TnC by preferentially perturbing *N*-terminal structure.

REFERENCES

Allen, T. S., L. D. Yates, and A. M. Gordon. 1992. Ca^{2+}-dependence of structural changes in troponin-C in demembranated fibers of rabbit psoas muscle. *Biophys. J.* 61:399–409.

Brandt, P. W., D. Roemer, and F. H. Schachat. 1990. Co-operative activation of skeletal muscle thin filaments by rigor cross-bridges. *J. Mol. Biol.* 212:473–480.

Bremel, R. D., and A. Weber. 1972. Cooperation within actin filaments in vertebrate skeletal muscle. *Nat. New Biol.* 238:91–101.

Corrie, J. E. T., and J. S. Craik. 1994. Synthesis and characterization of pure isomers of iodoacetamidotetramethylrhodamine. *J. Chem. Soc., Perkins Trans.* 1:2967–2974.

Dantzig, J. A., and Y. E. Goldman. 1985. Suppression of muscle contraction by vanadate. Mechanical and ligand binding studies on glycerol-extracted rabbit fibers. *J. Gen. Physiol.* 86:305–327.

Dong, W., S. S. Rosenfeld, C. K. Wang, A. M. Gordon, and H. C. Cheung. 1996. Kinetic studies of calcium binding to the regulatory site of troponin C from cardiac muscle. *J. Biol. Chem.* 271:688–694.

Dong, W., C. K. Wang, A. M. Gordon, S. S. Rosenfeld, and H. C. Cheung. 1997. A kinetic model for the binding of Ca^{2+} to the regulatory site of troponin from cardiac muscle. *J. Biol. Chem.* 272:19229–19235.

Dong, W., J. Xing, M. Villain, M. Hellinger, J. M. Robinson, M. Chandra, R. J. Solaro, P. K. Umedal, and H. C. Cheung. 1999. Conformation of the regulatory domain of cardiac muscle troponin C in its complex with cardiac troponin I. *J. Biol. Chem.* 274:31382–31390.

Farah, C. S., and F. C. Reinach. 1995. The troponin complex and regulation of muscle contraction. *FASEB. J.* 9:755–767.

Fuchs, F. 1977. The binding of calcium to glycerinated muscle fibers in rigor. The effect of filament overlap. *Biochim. Biophys. Acta.* 491:523–531.

Fuchs, F., and Y.-P. Wang. 1991. Force, length, and Ca^{2+}-troponin C affinity in skeletal muscle. *Am. J. Physiol. Cell Physiol.* 253:C541–C546.

Geeves, M. A., and S. S. Lehrer. 1994. Dynamics of the muscle thin filament regulatory switch: the size of the cooperative unit. *Biophys. J.* 67:273–282.

Gordon, A. M., E. Homsher, and M. Regnier. 2000. Regulation of contraction in striated muscle. *Physiol. Rev.* 80:853–924.

Gulati, J., E. Sonnenblick, and A. Babu. 1991. The role of troponin C in the length dependence of Ca^{2+}-sensitive force of mammalian skeletal and cardiac muscles. *J. Physiol. (Lond.).* 441:305–324.

Hannon, J. D., P. B. Chase, D. A. Martyn, L. L. Huntsman, M. J. Kushmerick, and A. M. Gordon. 1993. Calcium-independent activation of skeletal muscle fibers by a modified form of cardiac troponin C. *Biophys. J.* 64:1632–1637.

Hannon, J. D., D. A. Martyn, and A. M. Gordon. 1992. Effects of cycling and rigor cross-bridges on the conformation of cardiac troponin C. *Circ. Res.* 71:984–991.

Hazard, A. L., S. C. Kohout, N. L. Stricker, J. A. Putkey, and J. J. Falke. 1998. The kinetic cycle of cardiac troponin C: calcium binding and dissociation at site II trigger slow conformational rearrangements. *Protein Sci.* 7:2451–2459.

Hofmann, P. A., and F. Fuchs. 1987a. Effect of length and cross-bridge attachment on Ca^{2+}-binding to cardiac troponin C. *Am. J. Physiol. Cell Physiol.* 253:C90–C96.

Hofmann, P. A., and F. Fuchs. 1987b. Evidence for a force-dependent component of calcium binding to cardiac troponin C. *Am. J. Physiol. Cell Physiol.* 253:C541–C546.

Johnson, J. D., J. H. Collins, S. P. Robertson, and J. D. Potter. 1980. A fluorescent probe study of Ca^{2+}-binding to the Ca^{2+}-specific sites of cardiac troponin and troponin C. *J. Biol. Chem.* 255:9635–9640.

Kentish, J. C., and S. Palmer. 1989. Calcium binding to isolated bovine cardiac and rabbit skeletal troponin-C is affected by pH but not by caffeine or inorganic phosphate. *J. Physiol.* 417:160P.

Lehrer, S. S. 1994. The regulatory switch of the muscle thin filament: Ca^{2+} or myosin heads? *J. Muscle Res. Cell Motil.* 15:232–236.

Leszyk, J., R. Dumaswala, J. D. Potter, and J. H. Collins. 1988. Amino acid sequence of bovine cardiac troponin I. *Biochemistry.* 27:2821–2827.

Li, M. X., L. Spyracopoulos, and B. D. Sykes. 1999. Binding of cardiac troponin-I$_{147–163}$ induces a structural opening in human cardiac troponin C. *Biochemistry.* 38:8289–8298.

Martyn, D. A., P. B. Chase, and J. D. Hannon. 1993. Activation dependence of unloaded shortening velocity in skinned skeletal muscle fibers studied with activating troponin C. *Biophys. J.* 64:135a (Abstr.).

Martyn, D. A., C. J. Freitag, P. B. Chase, and A. M. Gordon. 1999. Ca^{2+} and cross-bridge-induced changes in troponin C in skinned skeletal muscle fibers: effects of force inhibition. *Biophys. J.* 76:1480–1493.

McKillop, D. F. A., and M. A. Geeves. 1993. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys. J.* 65:693–701.

Metzger, J. M. 1995. Myosin binding-induced cooperative activation of the thin filament in cardiac myocytes and skeletal muscle fibers. *Biophys. J.* 68:1430–1442.

Pan, B.-S., and R. J. Solaro. 1987. Calcium-binding properties of troponin C in detergent-skinned heart muscle fibers. *J. Biol. Chem.* 262:7839–7849.

Putkey, J. A., D. G. Dotson, and P. Mouawad. 1993. Formation of inter and intramolecular disulfide bonds can activate cardiac troponin C. *J. Biol. Chem.* 268:6827–6830.

Putkey, J., W. Liu, X. Lin, S. Ahmed, M. Zhang, J. D. Potter, and W. G. Kerrick. 1997. 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. *Biochemistry.* 36:970–978.

Sheng, Z., B. S. Pan, and J. D. Potter. 1992. Isolation, expression and mutation of a rabbit skeletal muscle cDNA clone for troponin I. The role of the NH2 terminus of fast skeletal muscle troponin I in its biological activity. *J. Biol. Chem.* 267:25407–25413.

Sia, S. K., M. X. Li, L. Spyracopoulos, S. M. Gagne, W. Liu, J. A. Putkey, and B. D. Sykes. 1997. Structure of cardiac muscle troponin C unexpectedly reveals a closed regulatory domain. *J. Biol. Chem.* 272:18216–18221.

Smith, C. A., and I. Rayment. 1995. X-ray structure of the magnesium(II) · ADP · vanadate complex of the *Dictyostelium discoideum* myosin motor domain to 1.9 Å resolution. *Biochemistry.* 35:5404–5417.

Solaro, J., and H. M. Rarick. 1998. Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. *Circ. Res.* 83:471–480.

Spyracopoulos, L., M. X. Li, S. K. Sia, S. M. Gagne, M. Chandra, R. J. Solaro, and B. D. Sykes. 1997. Calcium-induced structural transition in the regulatory domain of human cardiac troponin C. *Biochemistry.* 36:12138–12146.

Strauss, J. D., C. Zeugner, J. E. Van Eyk, C. Bletz, M. Troschka, and J. C. Rüegg. 1992. Troponin I replacement in permeabilized cardiac muscle. Reversible extraction of troponin I by extraction with vanadate. *FEBS Lett.* 310:229–234.

Tanner, J. W., D. D. Thomas, and Y. E. Goldman. 1992. Transients in orientation of a fluorescent cross-bridge probe following photolysis of caged nucleotides in skeletal muscle fibres. *J. Mol. Biol.* 223:185–203.

Tobacman, L. S. 1996. Thin filament-mediated regulation of cardiac contraction. *Annu. Rev. Physiol.* 58:447–481.

Tripet, B., E.-J. E. Van, and R. S. Hodges. 1997. Mapping of a second actin-tropomyosin and a second troponin C binding site within the C terminus of troponin I, and their importance in the Ca^{2+}-dependent regulation of muscle contraction. *J. Mol. Biol.* 271:728–750.

Vibert, P., R. Craig, and W. Lehman. 1997. Steric model for activation of muscle thin filaments. *J. Mol. Biol.* 266:8–14.

Wang, Y. P., and F. Fuchs. 1994. Length, force, and Ca^{2+}-troponin C affinity in cardiac and slow skeletal muscle. *Am. J. Physiol. Cell Physiol.* 266:C1077–C1082.

Wang, Y. P., and F. Fuchs. 1995. Osmotic compression of skinned cardiac and skeletal muscle bundles: effects on force generation, Ca^{2+} sensitivity and Ca^{2+} binding. *J. Mol. Cell. Cardiol.* 27:1235–1244.

Wilkinson, J. M., and R. J. A. Grand. 1975. The amino acid sequence of troponin I from rabbit skeletal muscle. *Biochem. J.* 149:493–496.

Zot, H. G., and J. D. Potter. 1987. Calcium binding and fluorescence measurements of dansylaziridine-labeled troponin C in reconstituted thin filaments. *J. Muscle Res. Cell Motil.* 8:428–436.